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GRANT NUMBER DAMD17-94-J-4028

TITLE: Expression and Regulation of the Retinoic Acid Receptor Beta Gene in Human Mammary Epithelial Cells

PRINCIPAL INVESTIGATOR: Karen Swisshelm, Ph.D.

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98195

REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (22 Aug 95 - 21 Aug 96)

4. TITLE AND SUBTITLE Expression and Regulation of the Retinoic Acid Receptor Beta Gene in Human Mammary Epithelial Cells

5. FUNDING NUMBERS
DAMD17-94-J-4028

6. AUTHOR(S)
Karen Swisshelm, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
Seattle, WA 98195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only; proprietary information, Sep 96. Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We have performed ßRARE (beta retinoic acid response element) gel shifts and supershifts, using antibodies for RARα and RARß, with probes to nuclear extracts from 2 normal and 3 tumor lines. We have performed these analyses with RARß-transduced breast cancer cells, MCF7 and MDA-MB-231. From 13 breast cancer cell lines, we have PCR-amplified four overlapping regions of the RARß promoter (totaling ~700 ntds) and performed primary sequence analysis. We have studied the effects of retinoic acid (RA) on growth of normal human mammary epithelial cells (HMECs) in comparison to tumor cells. We have begun experiments to examine the *in vivo* behavior of breast cancer cell lines transduced with the RARß gene through nude mice studies. By differential display, we have identified eight genes regulated by retinoic acid in either breast cancer cell lines and/or normal HMECs. We have continued to evaluate the methodology to assess RAR levels in primary breast tumors. Our studies underscore that a rich variety of cellular processes are regulated by RA in concert with RARß in normal and neoplastic mammary cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer; Retinoic Acid; Growth Control	83
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	limited

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Karen Swisshelm 9/16/96

PI - Signature Date

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Introduction

Breast Cancer May be Prevented by Retinoids:

Retinoid therapy or dietary intervention may offer new avenues of breast cancer prevention or therapy. Clinical trials in the U.S. (National Cancer Institute) and Italy (Milan) are now determining the efficacy of retinoid chemotherapeutics[1] . The importance of retinoids in the *prevention* of breast cancer is strongly suggested based on the Harvard School of Public Health nurses' cohort study(n > 89,000), where it is indicated that moderate dietary intake of carotenoids (plant source of vitamin A) as well as preformed vitamin A is inversely associated with the risk of breast cancer (Rev in: 2). *Note:* this previous study is ungoing and will likely reveal additional trends given longitudinal nature of the study. Additional support for retinoid therapeutics derives from a large body of animal carcinogenesis studies where synthetic retinoids were found to reduce mammary tumor incidence in mice and rats [Rev. in: 3].

Retinoids, including vitamin A and one of its metabolites, all-*trans* retinoid acid (RA), are a class of over 3000 naturally occurring and synthetic compounds. They were first recognized as potential cancer preventive substances 70 years ago when it was observed that epithelium of vitamin A deficient animals resembled that of preneoplastic tissue [4]. Humans obtain retinoids as dietary constituents (usually carotinoids or pre-formed vitamin A) that are metabolized in the intestine, the liver, as well as in "target" tissues such, as the skin, lymphocytes, lung and breast, where it hypothesized they maintain a differentiated phenotype. Esterified retinoids are stored in the liver and may accumulate in organs with high fat content such as the mammary gland [5, 6]. The most widely studied of the retinoid metabolites is retinoic acid (RA). Retinol or precursors of retinoic acid are metabolized *in vivo* to an active metabolite, *all-trans* retinoic acid (AT-RA, often abbreviated to RA). Currently a number of clinical trials are underway using retinoids (natural and synthetic), either alone or in combination with other therapies to treat a variety of cancers, including breast cancer [1]. Our studies of one of the nuclear receptors for retinoic acid, RARß [retinoic acid receptor beta], are based on the paradigm that the action of retinoids in concert with expression of tissue-appropriate wild-type nuclear retinoic acid receptors promotes a differentiated phenotype in breast epithelial cells, and in particular in the luminal epithelial cells where most human breast tumors arise.

Proteins That Participate in Retinoid Action:

Although we are targeting one nuclear RAR for the focus of our studies, we must keep in mind for both our current and future studies the larger picture of retinoid action. Three classes of intracellular proteins may play roles in the phenotypic response of target cells (normal vs. tumor) to retinoids:

a) the circulating retinol binding proteins and cytosolic retinol and retinoic acid binding proteins [CRBPs; CRABPs]; and

b) the nuclear, retinoic acid receptors [RARs; RXRs]

c) the enzymes involved in the transformation of retinol to retinoic acid;

Very little is known about the intracellular proteins which metabolize retinoids and their relationship to cancer risk or prevention. However, it is interesting to note that several of these proteins involved in retinoid uptake and action are themselves regulated at the transcriptional level by retinoic acid, e.g. RARß and CRBP1.

Much less is known about the expression and regulation of the CRBPs or CRABPs in relationship to breast cancer. A recent study reported expression of CRABPII (but not I) in both RA responsive (growth suppressed) and RA resistant breast cancer cell lines [7]. Interestingly, *in vitro*, both RARß and CRABPII are upregulated at the mRNA level by retinoic acid. These proteins are likely to be important in sequestering retinol or retinoic acid derivatives and for delivery to subcellular regions (microsomes) where the ligands are metabolized.

A striking number of neoplasias exhibit either direct or indirect involvement of the retinoic acid receptors. The theme in most cases is either a rearrangement in the normal gene or loss of expression. The majority of cases of acute promyelocytic leukemia, for example, exhibit a characteristic translocation between chromosome 15 and 17 (the site for the RARα gene). Promyelocytic leukemia patients frequently attain remission with retinoids presumably because there is sufficient wild type RARα in the neoplastic cells to respond and to overcome the transforming, aberrant protein. Recently it was demonstrated that in treatment of patients with "premalignant" oral neoplasia with 13-*cis*-retinoic acid, the expression of the RARß gene was specifically up-regulated and this was associated with "clinical response"[8]

Loss of expression of the retinoic acid receptor *beta* (RARß) has been documented, to date, in cells derived from the following solid tumors: ovarian cancer [9]; lung cancer [10, 11] squamous cell carcinoma [12] head and neck cancer [13] gastric cancer [14] and breast cancer [15]. This latter paper was work I performed that established, in part, the basis for this Army-funded research. The RARß gene has properties of a tumor suppressor gene; it slows growth of lung tumors in nude mouse assays [11]and, when transfected, causes breast cancer cells to undergo apoptotic cell death[16, 17] Growth suppression of breast tumor cells may also be mediated through RARα and RARγ [18, 19] , although no transfection or in vivo experiments strongly support RARα and RARγ as tumor or growth suppressors, as is the case for RARß. There is a strong interest both in academic medicine and in the pharmaceutical industry to design retinoic acid derivatives to target specific receptors and to study these a specific chemotherapeutics specifically for breast cancer (information this P.I. obtained from **attendance at a recent FASEB meeting on Retinoids, Copper Mountain, CO., June 23-28, 1996** (also recent refs: 20-22).

Our major, current goals, based on two key papers [15, 16], are to confirm the hypothesis that RARß is a tumor suppressor gene for breast cancer and to understand the receptor and ligand interactions necessary for invoking and maintaining growth control and/or a differentiated phenotype.

Our broad, future goals include an understanding of the retinoid metabolic pathways, the genes involved in this process, and genes that may transcriptionally modulated by retinoic acid or metabolites of retinoic acid in the mammary gland.

Overview: This progress report addresses four areas of work accomplished over the past year:

1) We have made progress in sequencing and analysis of the RARß gene promoter *(Task 1)*. We have advanced our studies of protein:DNA interactions within the RARß gene promoter in breast cancer cells *(Task 1)*.

2) We have made progress on Task 2 by furthering our understanding of RA growth control in normal HMECs*(see Appendix manuscript)*. We have identified an extracellular matrix protein that is regulated by RA in normal cells and RARß-transduced breast cancer cells MDA-MB-231. We have established the base line studies for animal (nude mouse) experiments.

3) We have made progress in cloning 5 genes by differential display (positive selection by retinoic acid induction in breast tumor cells, MCF7M) and cloned near full length of 2/5. Using the DD technology, we have identified three genes upregulated by retinoic acid in normal human mammary epithelial cells *(Task 3)*.

4) We have critically evaluated state of the art methodology to assess levels of RARß gene expression in primary tumors, and have begun work on *in situ* hybridization and reverse transcriptase- polymerase chain reaction (RT-PCR), using breast tumor cell lines which do or do not express RARß mRNA *(Task 4)*.

Body:

1) **Analysis of RARß promoter (Task 1)**

a) ***DNA: protein interactions.*** We have utilized both the wild type and mutant synthetic, double-stranded ßRARE DNA probes, with a direct repeat separated by 5 nucleotides in gel shift assays:

ßRAREs	
WT	GGGTAGGGTTCACCGAAAGTTCACTCG
Mutant:	GGGTAGGCTTACCCGAAAGTTCACTCG
*= non-identity	* **

And, as an additional negative control, wild type and mutant DNA probes for a consensus **RXRE,** with a direct repeat separated by 1 nucleotide:

RXREs	
WT	CTCTGCAGGTCACAGGTCACTTTTCCT
Mutant:	CTCTGCACGTACCAGGTCACTTTTCCT
*= non-identity	* **

to test the specificity and sensitivity of DNA:nuclear protein interactions in normal, tumor, and RARß gene-transduced breast cancer cells [16]. We have also assayed the specificity of the DNA:protein complexes using RARα-, RXRß- and RARß-specific antibodies in gel super-shift assays. To address the possibility that other known proteins that "cross talk" with the nuclear retinoic acid receptors are involved in aberrant DNA:protein:protein complexes involving the ßRARE in breast tumor cells, we initiated gel shift and supershift assays using AP1 DNA probes and an antibody to the early response/transcription factor, c-jun.

We have tested the following nuclear extracts from cells grown in the presence or absence of RA with ^{32}P-labeled probes:

- 2 normal HMEC strains
- 3 breast cancer cell lines: MCF7, MDA-MB-231, and Hs578T

•2 series of clones and controls of RARß-transduced breast cancer cells, MCF7 and MDA-MB-231

Results:

•Both the RARα and RARß antibodies caused a supershift in DNA:protein complexes when the target DNA is wild type ßRARE. This was observed in both normal and tumor cells

•RXRE complexes are of a lower (less dense) mobility for normal HMECs compared to tumor cells

•MDA-MB-231 cells (parentals, controls, and RARß-transduced) exhibit diminished binding to the RXRE in cells cultured in RA compared to MCF7 RARß-transduced cells and controls.

•Both MCF7- and MDA-MB-231- RARß-transduced cells exhibit abundant supershift complexes specifically with an antibody to RARß

b) ***Sequence analysis of the RARβ promoter in breast cancer cell lines:*** In addition to the three primer sets discussed in last year's progress report, we have utilized an additional primer set (IV).

Primer set I (Region B)*	forward:	5'- AGGAGCAGCGTCCCGGC-3'
	reverse	5'-CCTACTACTTCTGTCAC-3'
		inclusive for nucleotides -262 to +16 relative to the start of transcription:
Primer set II (Region A)	forward:	5'- GTGGCCTGTGTGTTTGGGAC-3'
	reverse	5'-CTCGCAGTGTAGAAATCCAGG-3'
		inclusive for nucleotides -721 to -464 relative to the start of transcription
Primer set III (Region D)	forward:	5'- GTGGCCTGTGTGTTTGGGAC-3'
	reverse	5'-CTCGCAGTGTAGAAATCCCAGG-3'
		inclusive for nucleotides -721 to + 535 relative to the start of transcription
Primer set IV (Region C)	forward:	5'- GTGGCCTGTGTGTTTGGGAC-3'
	reverse	5'-CCTACTACTTCTGTCAC-3'
		inclusive for nucleotides -721 to +17 relative to the start of transcription

*See also Appendix I for "regions"

Thus we will be able to compare and verify the sequences of overlapping fragments for each cell line.

We have extracted genomic DNA from 13 breast cancer cell lines and one normal cell line. In the majority of these lines, we extracted DNA from cells received from different sources. For example we have MCF7 cells from ATCC, the Michigan Cancer Foundation, and the Dana-Farber Cancer Institute. Lacking the ability to test differently derived cell sources, we have also obtained DNA from different culture passages in order to be confident that any changes we may see in the sequence of the promoter were likely present in the initially isolated tumor sample or at an early passage in our laboratory. We have designed and

implemented experiments to amplify by PCR each of the four specific regions of the promoter for 33 DNA purifications. PCR amplicons were detected by ethidium bromide staining of 1.8% agarose gels containing electrophoresed- PCR reactions and DNA markers for size determination. DNA bands were excised by a sterile razor blade and purified over a micro concentrator purification column (Amicon). In several instances, two bands were visualized for one PCR, depending on the cell line. In those cases, both bands were excised. Automated fluorescent sequencing was performed by J. Strange, Murdock Laboratory, Univ. of Montana, using one or both of the primers used in the PCR amplification. Currently we are analyzing each region for homology to the published sequence of the RARß promoter. using a subroutine, Pileup, in GCG (a sequence analysis package, Genetics Computer Group, Madison, WI) on the mainframe computer, McClintock at the University of Washington.

Results:
68 sequences from 13 breast cancer cells are under analysis. Regions of mismatch or gaps are examined on the chromatogram for that sequence. In several cases, we anticipate having to re-amplify and sequence manually. **See Appendix I for an example of a sequence alignment.**

c) Luciferase reporter gene constructs for studying the specific negative regulatory elements utilized by breast tumor cells. We have amplified constructs and verified restriction fragments/inserts for the following RARß promoter luciferase reporter constructs, all of which were placed in the pGL2 basic vector (Promega):

construct name:	Size of promoter frag. & element(s)*
pL-015	1.655 kb includes TRE, CRE, & ßRARE
pL-016	0.902 - includes TRE, CRE, & ßRARE
pL-017	0.677 -includes TRE, CRE, & ßRARE
pL-018	0.411 -includes TRE, CRE, & ßRARE
pL-019	0.398 - includes TRE, CRE, & ßRARE
pL-020	0.279 - includes TRE, CRE, & ßRARE
pL-021	0.233 - TRE + ßRARE
pL-022	0.215 -wt ßRARE
pL-023	0.233 - mtTRE + ßRARE
pL-025	0.268 - mtCRE + ßRARE
pL-026	0.305 - CRE+TRE+mtßRARE
pL-027	0.305 - CRE+mtTRE+mtßRARE

* TRE = thyroid hormone response element; CRE = cAMP-response element; ßRARE = retinoic acid response element. All of these elements are found the promoter the RARß gene. Constructs originated by Hui Tsou, Columbia University.

Following transient transfection with lipofectin (Gibco, BRL), five of these constructs (-015; -018, -019, -020, and -021) have been tested for luciferase activity in RARß-transduced tumor lines along with positive and negative control plasmids, pGL2 control and pGL2basic. We feel confident that we can perform similar experiments in tumor cell lines.

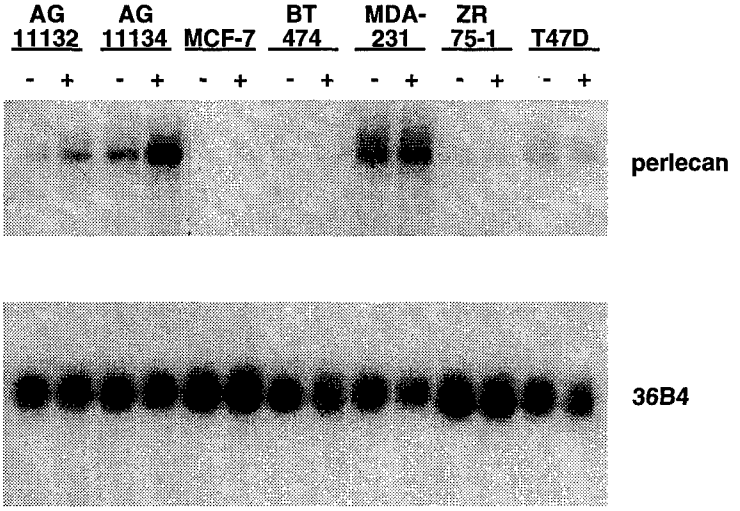
2) **Functional RARß gene in breast tumor cells and phenotypic changes (Task 2):** We have introduced the full length human RARß gene into four breast tumor cell lines: MCF7, MDA-MB-231, MDA-MB-435, and BT 474 (*Cell Growth and Differentiation* 6:1077-1088, September 1995).

We have begun a phenotypic characterization of the RARß-transduced cells. We observed that the transduced tumor cells expressing RARß constitutively exhibited a more flattened adherent morphology in culture. We questioned if this was a direct or indirect result of reconstituted RARß expression. In order to begin to answer this issue, we examined normal HMECs in order to determine if they, too, underwent apoptosis as the RARß-transduced breast tumor cells did when cultured in RA. We found, in contrast, that normal HMECs failed to undergo apoptosis, but instead underwent a state of growth arrest. This distinguishing result between normal and tumor cells is quite important in light of the possible chemotherapeutic use of retinoid compounds (**see Manuscript, Appendix II**) e.g., if normal cells are preserved, while tumor cells are selectively killed.

We have examined a number (10) of down-stream candidate genes (both oncogenes, cell cycle regulatory genes, and genes that might affect cell morphology) for altered expression as a consequence of the introduction of the RARß-gene.

The one candidate that exhibited altered regulatory expression when normal or tumor cells are cultured in RA was the extracellular matrix molecule gene, ***perlecan*** **(see attached, Figure 1, 2 and 3)** Upregulation of perlecan is significant for several aspects of cellular physiology: a) perlecan may serve as a reservoir for positive or negative regulatory growth factors; and b) perlecan deposition is important in maintaining an intact basement membrane [23]. Other investigators have examined perlecan in primary breast cancers and find that more advanced tumor beds exhibited loss of perlecan protein accompanying a loss of intact basement membrane [24].

Figure 1



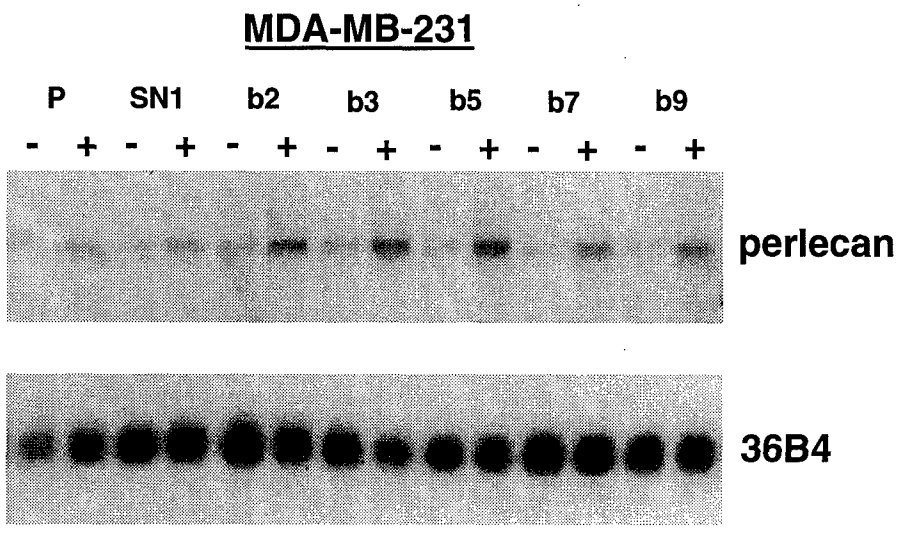
Expression of the perlecan gene in normal and breast cancer cells in response to culture in retinoic acid. Cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μM retinoic acid. Total RNA was collected and Northern blot analysis was performed. The blot was first probed with a cDNA for perlecan [25] and subsequently with a control probe, 36B4 (see [15, 16] for details of growth, RNA collection and Northern analysis).

Results summary: Perlecan exhibits up-regulation by retinoic acid in two normal HMEC strains, AG-11132 and AG-11134. It lacks appreciable expression in 4 estrogen receptor positive breast tumor cell lines, MCF-7, BT-474, ZR-75-1, and T47D. Perlecan exhibits up regulation in the estrogen receptor negative breast cancer line, MDA-MB-231.

Significance: Elaboration of basement membrane protein perlecan may be down-regulated or altered, particularly in estrogen receptor positive tumors.

Note: this work (and the results shown in Figures 2 and 3) is the result of a collaborative effort among Drs. Michael Kinsella (a proteoglycan expert in the Dept. of Pathology at the University of Washington) and Victoria Seewaldt (Department of Medical Oncology, University of Washington).

Figure 2



Expression of the perlecan gene RARß-transduced breast cancer cell line, MDA-MB-231. Cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μM retinoic acid. Total RNA was collected and Northern blot analysis was performed. The blot was first probed with a cDNA for perlecan as described in Figure 1. P = parental line; SN1 = one vector-alone clone; b2, b3, b5, b7, b9 = five independent clones of MDA-MB-231 transduced with a retroviral vector containing the human RARß gene (normally MDA-MB-231 cells do not express RARß mRNA.

Results summary: MDA-MB-231 cells transduced with the RARß gene exhibit enhanced expression (about 10-fold) of the perlecan gene when cells are cultured in RA.

Significance: The basement membrane protein perlecan is a likely downstream gene whose expression is regulated by retinoic acid through transcriptional activation of the RARß gene. Activation of the perlecan gene is predicted in an *in vivo* model of breast cancer (nude mouse studies).

Figure 3

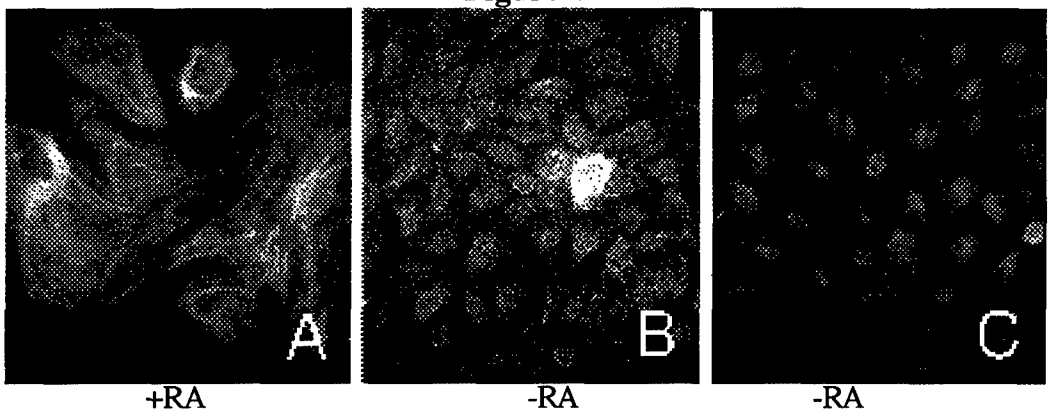


Fig. 3. Perlecan protein deposition is facilitated by RA. Normal HMEC strain, AG-11132 was plated onto slides coated with gelatin and cultured for 3 weeks in the absence of RA. Panel A: HMECs cultured in RA for four days. Panels B and C: HMECs cultured for four days in the absence of RA. All cells were fixed cold in 3% paraformaldehyde, followed by ice cold methanol fixation. Immunocytochemistry was performed with a fluorescence labeled- perlecan antibody [α PCab](1:500 dilution) to cells shown in panels A and B (EY9; rabbit anti-mouse; John Hassell, University of Pennsylvania). Panel C was incubated with normal rabbit serum [NRS].

Results: Normal cells cultured in RA exhibit a distinct alteration in the extracellular matrix as a result of perlecan protein expression, as evidenced by antibody staining in situ.

In order to further characterize the RARß-transduced cells we have initiated nude mouse studies to determine if breast cancer cells that contain the reconstituted RARß gene show altered tumor growth kinetics *in vivo*. Our first experiments were initiated this summer. We inoculated 4 animals each with 10^6 or 10^5 MDA-MB-435 breast tumor cells as well as sham (media) control into the mammary fat pad of Balb/c nu/nu female mice. We have obtained experimental design advice from Dr. Janet Price, University of Texas, M.D. Anderson Cancer Center, Houston, TX [26] . Our initial experiment was performed in order to determine our success in animal-survival during surgery (100% survived) as well as the time for progression of tumor "take". Animals were sacrificed at 9 weeks and necropsy was performed. We have been very fortunate to have the expert advice and scientific collaboration of investigators from the Comparative Medicine at the University of Washington, Drs. Lillian Price and Ted Birkebak. Dr. Price assisted in the surgeries and in the monitoring of the animals in a specific pathogen free facility. Dr. Birkebak, a veterinary pathologist assisted us in necropsy.

In our next experiment we will test if the RARß-transduced cells MDA-MB-435 cells exhibit a different biologic course in the nude mouse. We anticipate no difficulties in fulfilling this task.

3) **Determination of genes expressed as a consequence of culture in retinoic acid (Task 3):** We have continued our studies to detect differences in gene expression in cells cultured in the differentiating agent, retinoic acid. We have used the method of differential display (DD) of mRNA [6] to identify genes regulated by RA. The tables below show our progress in two cell models. The first (Table 1) summarizes our work with MCF7 breast cancer cells; Table 2 summarizes our work with normal HMECs (AG11132 cells). In both models we selected primarily potential mRNAs that were up-

regulated upon RA culture. *In summary, we have 4 genes of known function and , at this time in the sequencing strategy, 4 novel genes that exhibit enhanced transcription with RA.*

Differential Display summary I:

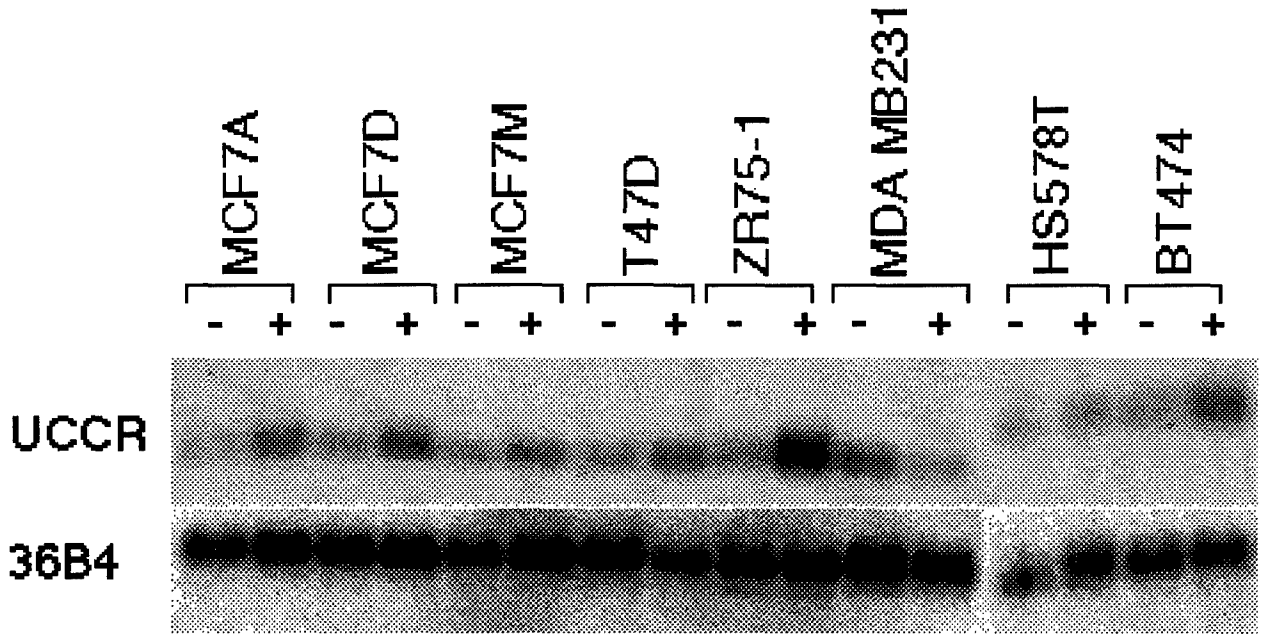
Selection of DD PCR products with RA-associated Enhanced Expression in MCF7 breast cancer cells

Status	Number
Number of primer combinations tested	25
Bands eluted from DD gel	34
Positive candidates on screening Northern	16
Gene candidates cloned + positive on second Northern screen	13
Gene candidates partially sequenced	5
Sequenced candidates for continued studies	5
Candidates for functional studies	1
Candidates to be cloned	1

We have elected to further investigate the functional properties one candidate, which exhibits approximately 4-5-fold increased expression in MCF7 cells cultured in 1 μM retinoic acid for 48 hours. This gene encodes for a protein related to a ubiquinol-cytochrome C reductase 9.5 kD polypeptide (UCCR). This peptide likely resides in the mitochondria inner space and participates in electron/proton pumping, and ultimately the energy metabolism of cells. We have screened a cDNA library prepared from ZR-75-1 cells (Clontech) and obtained plaques containing various lengths of this gene. The longest clone is ~700 nucleotides, which we believe contains the full-length cDNA sequence, based on the published bovine sequence. The human gene has not been reported. We have begun studies using FACS analysis and mitochondria-specific dyes to ascertain if RA effects electron transport.

Examples of the expression pattens for a sample of these above genes or gene candidates is shown in Figures 4 and 5 below.

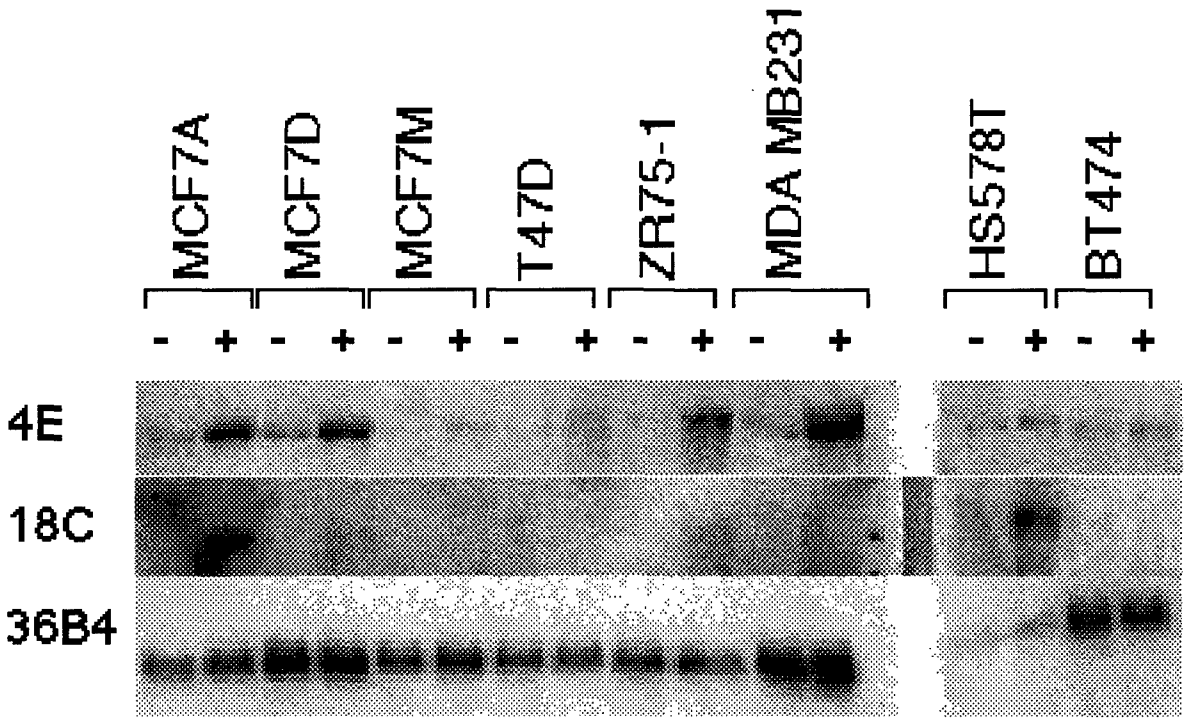
Figure 4



Expression of the human 9.5 kD ubiquinol-cytochrome C reductase (UCCR) gene in breast cancer cells in response to culture in retinoic acid. All cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μM retinoic acid. As with Figures 1 and 2, total RNA was collected and Northern blot analysis was performed with 10 μg of total RNA. The blot was first probed with the cloned differential display amplified product and subsequently with a control probe, 36B4 (see previous figures for references on the details of Northern analysis).

Results summary: The expression of the UCCR gene, encoded in the nucleus, with the gene product utilized in complex III of electron transport in the mitochondria, is upregulated in 5/6 breast tumor cell lines. Note: we tested three independent isolates of MCF7, denoted M, D, and A (M = Michigan Cancer Research Foundation; D = Dana-Farber Cancer Institute, Boston; and A = American Type Culture Collection.)

Figure 5



Expression of two genes identified by differential display in breast cancer cells in response to culture in retinoic acid. All cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μM retinoic acid. As with Figures 1 and 2 and 4, total RNA was collected and Northern blot analysis was performed with 10 μg of total RNA. The blot was first probed with the cloned differential display (DD) amplified products (4E and 18C) and subsequently with a control probe, 36B4 (see previous figures for references on the details of Northern analysis).

Results summary: The expression results from two cloned DD-PCR products which have been seqeunced and found to be novel. Both genes are upregulated by RA in MCF7M cells, and to various extents expressed and upregulated in other breast cancer cell lines. They are both novel in that neither encodes for a known gene, but they both have been cloned and submitted to GenBank as anonymous, EST (expressed seqeuence tagged) seqeunces. Note: This is a different blot from Figure 4. We tested three independent isolates of MCF7, denoted M, D, and A (M = Michigan Cancer Research Foundation; D = Dana-Farber Cancer Institute, Boston; and A = American Type Culture Collection.)

Differential Display summary II:

Selection of DD PCR products with RA-associated Enhanced Expression in *Normal* HMECs, AG11132

Status	Number
Number of primer combinations tested	33
Bands eluted from DD gels	9
Positive candidates on screening Northern	8
Gene candidates cloned	3
Gene candidates partially sequenced	3*
Library screening	N/D

*One candidate encodes a human pre-mRNA splicing factor 2 p32; the second candidate encodes the human dihydrodiol dehydrogenase; and the third shows homology to human clordecone reductase by Blast algorithm searches (World Wide Web databases and GCG).

4) **Initiation of studies to determine quantitative and qualitative expression of the RARß gene in vivo (Task 4).**
We have made the least progress on this task during the past year, due primarily to technical difficulties and irreproducibility of experiments, based on the techniques of Ferrari et al. [27]. In situ experiments have been even more challenging. We have plans for specific changes in procedures or tactics.

a) We have been unable to amplify reproducibly the RARß gene. We are currently revising our strategy for semiquantitative PCR of the RARs from tissue samples, by designing different primers.

b) We have found the RNA:RNA in situ experiments to be problematic in obtaining consistent, repeatable results. We find that tissue from breast biopsy specimens (formalin-fixed and paraffin embedded) to be exquisitely sensitive to Proteinase K digestion. This observation was confirmed to us by Dr. Rubin Lotan's group (discussions at recent FASEB Retinoid meeting).

We have continued to bank breast biopsy specimens and currently have accrued samples from over 140 individuals.

Conclusions:

1. Nuclear extracts from RARß-transduced breast cancer cells exhibit high levels of protein binding, specifically involving RARß protein, to the wild type ßRARE. We also have demonstrated RARß-transduced breast cancer cells show differential protein binding to an RXRE consensus. We preliminarily determined that approximately 50% of the tumor cell lines exhibit mutations/deletions in the RARß promoter. Full confirmation of this preliminary finding will lead us to a more intelligent analysis of potential mutation spectra analysis of the RARß gene in primary breast tumors.

2. We have demonstrated that one phenotypic sequela of breast cells cultured with retinoic acid is induction of the gene and gene product for perlecan, a heparan sulfate proteoglycan, deposited in the extracellular membrane space.

3. We have identified 9 genes that exhibit enhanced expression in either normal or breast tumor cells when cells are cultured in retinoic acid, identified either by "logic" or by differential display methodology.

Retinoic acid and the nuclear receptor, RARß, regulate a wide variety of cellular functions and biochemical pathways, from the mitochondria to the extracellular space, in order to maintain cellular homeostasis in mammary epithelial cells.

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Appendix I

Example of primary sequence analysis of the RARß promoter using the PILEUP algorithm in GCG (Genetics Computer Group).

Hierarchical identifiers include:

- #s 1-24 = coded cell line number
- A, B, C, or D = region of promoter
- F= forward primer
- i or ii = higher molecular weight (i) or lower molecular weight (ii) band excised from PCR gel.

The example shown here does not include the "wild type" RARß promoter sequence (GenBank identifier X56849).

PileUp of: @total.fil

Symbol comparison table: GenRunData:pileupdna.cmp CompCheck: 6876

GapWeight: 5.000
GapLengthWeight: .300

total.msf MSF: 433 Type: N September 18, 1996 09:16 Check: 3455 ..

Name: 5cCF	Len:	433	Check: 5055	Weight:	1.00
Name: 7cCF	Len:	433	Check: 2057	Weight:	1.00
Name: 14aiAF	Len:	433	Check: 176	Weight:	1.00
Name: 17aiAF	Len:	433	Check: 7737	Weight:	1.00
Name: 13ciCF	Len:	433	Check: 6273	Weight:	1.00
Name: 19CiiF	Len:	433	Check: 117	Weight:	1.00
Name: 19aiAF	Len:	433	Check: 511	Weight:	1.00
Name: 1CCF	Len:	433	Check: 9047	Weight:	1.00
Name: 23CiF	Len:	433	Check: 9818	Weight:	1.00
Name: 21CiF	Len:	433	Check: 9009	Weight:	1.00
Name: 8ciCF	Len:	433	Check: 4028	Weight:	1.00
Name: 2cCF	Len:	433	Check: 770	Weight:	1.00
Name: 3aAF	Len:	433	Check: 5286	Weight:	1.00
Name: 6aAF	Len:	433	Check: 4956	Weight:	1.00
Name: 18aiAF	Len:	433	Check: 9622	Weight:	1.00
Name: 24cCF	Len:	433	Check: 6732	Weight:	1.00
Name: 24aAF	Len:	433	Check: 4428	Weight:	1.00
Name: 22CiF	Len:	433	Check: 2612	Weight:	1.00
Name: 4cCF	Len:	433	Check: 58	Weight:	1.00
Name: 12ciiCF	Len:	433	Check: 496	Weight:	1.00
Name: 16ciiCF	Len:	433	Check: 1982	Weight:	1.00
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Name: 17ciiCF	Len:	433	Check: 9239	Weight:	1.00
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Name: 19DF	Len:	433	Check: 591	Weight:	1.00
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Name: 20BF	Len:	433	Check: 5184	Weight:	1.00
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Name: 13diDF	Len:	433	Check: 624	Weight:	1.00
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Name: 7diDF	Len:	433	Check: 8088	Weight:	1.00
Name: 1diDF	Len:	433	Check: 7636	Weight:	1.00
Name: 5diDF	Len:	433	Check: 598	Weight:	1.00

Name: 22Di	Len:	433	Check: 1929	Weight:	1.00
Name: 16dDF	Len:	433	Check: 4522	Weight:	1.00
Name: 23DiF	Len:	433	Check: 4888	Weight:	1.00
Name: 15dDF	Len:	433	Check: 2239	Weight:	1.00
Name: 19BF	Len:	433	Check: 226	Weight:	1.00
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Name: 21DiiF	Len:	433	Check: 7955	Weight:	1.00

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   7cCF  .........G GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  14aiAF ........TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  17aiAF ......GATG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  13ciCF ........TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  19CiiF ........TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  19aiAF ........TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
   1CCF  .......TAG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  23CiF  ........TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  21CiF  ........TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
   8ciCF  .......... GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
   2cCF  .........G GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
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   6aAF  .......... GCNTACTGGA ATAGGAATCT ATNNCACTCT GATNCCCCAC
  18aiAF ........TG GCAGACTGG. ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  24cCF  .cCF.seqTG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  24aAF  .........G GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
  22CiF  .........G GCAGACTGGA ATTGGAA.CT AGTTCAGTTT GATTCNCCAA
   4cCF  ......CTNG AAGAATTNNN ATGAANNTCT AGTTCATTGT NATACNCCNN
 12ciiCF ..........  ..........  ........CA TAGCAAGAAT TTAAAGTCCA
 16ciiCF ..........  ..........  ........CA TACCAAGAAT TTAAAGTCCA
 15ciiCF ..........  ..........  .......GCA TCCCAAGAAT TTAAAGTCCA
  23CiiF ..........  ..........  .......GCA TAGCAAGAAT TTAAAGTCCA
  20CiiF ..........  ..........  ........CA TAGCAAG.AN TTAAAGTCCA
  22CiiF ..........  ..........  .......GCA TAGCAAG.AN TTAAAGTCCA
 13ciiCF ..........  ..........  ........GC ATACAAG.AT TTAAAGTCCA
 17ciiCF ..........  ..........  ........CA TACCAAGAAT TTAAAGTCCA
  8ciiCF ..........  ..........  .........C CGNNCAAGAA TTANAGTCCA
   19DF  ..........  ..........  ........GC NCNGCAAGAT TTACAGTCCA
  17bBF  ..........  .......... CCCTCGCCCT CGCTCATTTT AANAGCNCTT
   3bBF  ..........  .......... .ACNCGCCCN CGCNCATNNA AACNNCNCTT
  24bBF  ..........  .......... GNCNCGCCCC AGCTCATNNG AANAGCNCCT
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   18bDF  .......... .......... ...CTCGCCC TGCTCATTTA AANANCACTT
   4diDF  .......... .......... ........CN CGCTCANTTT AANANCACTT
   7diDF  .......... .......... ........CT CGCTCATTTT AAAAGCNCTT
   1diDF  .......... .......... ....TCGCCC TGCTCATTTT AAAACCACTT
   5diDF  .......... .......... .........C NGCTCATTTT AAAACCACTT
    22Di  .......... .......... .CCCTCGCCC TGCTCATTTT AAAAGCACTT
   16dDF  .......... .......... ....CTCGCC TGCTCATTTA AAAAGCACTT
   23DiF  .......... .......... ..CCCTGGCC TGCTCATTTT AAAAGCACTT
   15dDF  .......... .......... .....GGACC CGCTCATNTN AANACCNCTT
    19BF  .......... .......... ......CCCT CNCTCATTTT AAAAGCNCNT
   19CiF  .......... .......... ......CCCT CNCTCATTTT AAAAGCNCNT
   24dDF  .......... .......... ...CTCGCCC TGCTCATTTT AAAAGCACTT
    2dDF  .......... .......... ...CTCGCCC AGCTCATTTT ACAAGCACTT
   8diDF  ..GAAAANNA NTNTNTTCAC CNCCCTGGAN TGCTTTTTTT ATATNCTTTT
 14aiiAF  .......... .......... .......... .......... ..........
 18aiiAF  .......... .......... .......... .......... ..........
 17aiiAF  .......... .......... .......... .......... ..........
 19aiiAF  .......... .......... .......... .......... ..........
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          51                                                  100
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    7cCF  CCCATGCTCT TGACCACTAT ACTGTATTTT CAAGTCCAGA TCTNAAATCT
  14aiAF  CCCATGCTCT TGACCACTAT ACTGTTTTTT CAAGTCCAGA TCTGAAATCT
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  13ciCF  CCCATGCTCT TGACCACTAT ACTGTTTTTT CAAGTCCAGA TCTGAAATCT
  19CiiF  CCCATGCTCT TGACCACTAT ACTGTTTTTT CAAGTCCAGA TCTGAAATCT
  19aiAF  CCCATGCTCN TNANCACTAT NCTGTTTTTT CAAGTCCAGA TCTGAAATCT
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   8ciCF  CCCATGCTCT TGACCACTAT ACTGTTTTTT CAAGTCCAGA TCTGAAATCT
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 17ciiCF  GTGGTTTGTC TTAGAGCCTG GACTCACACT GTGCAGTAAA TTTGATGCAA
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  19DF   GTGGTTTGTC TTAGAGCCTG GACTCACACT GTGCAGTAAA TTTGATGCAA
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  3bBF   CTTGTTTTGT TTCN.GAAGT NAAAAAAAAN AAACANCNCG CCGGCTNNTN
 24bBF   CTTGTATTGT TTGTAAANGT GAAAAAAAAG AAACAANNCG CCGGCTTGTG
 14bBF   CTTGTATTGT TTTT.AAGGT GAGAAAGAGN ANAGACCNCG CCGGCTTGTN
  20BF   CTTGTATTGT TTGT.AAAGT GAGAAAGAGN ANANANCGCN CCGGCTTGTC
 12diDF  CTTGTATTGT TTTT.AAGGT GAGAAATAAG AAAGAAAACG CCGGCTTGTG
 13diDF  CTTGTATTGT TTTT.AAGGT GAGAAATAAG AAANAAAACG CCGGCTTGTG
 18bBF   CTTGTATTGT TTNT.AAGGT GAGAAAAAAG AAANAANACG CCGGCTTGTG
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 23DiF   CTTGTATTGT TTTT.AAGGT GAGAAAGAGN ANAGAANNCG CCGGCTTGTN
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 19CiF   CTTNTATTGT TTGT.AAGGT GAGAAATAGG AAAGACCCCG CCGGCTTGTG
 24dDF   CTTGTATTGT TTTT.AAGGT GAGAAATAGG AAANAAAACG CCGGCTTGTG
  2dDF   CTTGTATTGT TTTTAACGTG AGAAATANGA AAGGAAAACG CCGGCTTGTG
 8diDF   CTTNTTTTGT TTTT.NNNGT GAGANANAAN AANNAACNCN CTNGCNTNTT
14aiiAF  .......... .......... .......... .......... ..........
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17aiiAF  .......... .......... .......... .......... ..........
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         101                                                 150
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 14aiAF  CATTTTCTGT GTGGCTGTGT GTTTGGGACA GGGGTAACCA ATTCCTGACT
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 13ciCF  CATTTTCTGT GTGGCTGTGT GTTTGGGACA GGGGTAACCA ATTCCTGACT
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 23CiF   CATTTTCTGT GTGGCTGTGT GTTTGGGACA GGGGTAACCA ATTCCTGACT
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  2cCF   CATTTTCTGT GTGGCTGTGT GTTTGGGACA GGGGTAACCA ATTCCTGACT
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 24aAF   CATTTTCTGT GTGGCTGTGT GTTTGGGACA GGGGTAACCA ATTCCTGACT
 22CiF   CATTTTCTGT GTGGCTGTGT GTTTGGGACA GGGGTAACCA ATTCCTGACT
  4cCF   CNNTNGCNGT GTGGCTGTGT GTCNGGGACA NGGGTNACCA NTTCCTGACT
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16ciiCF  TTTGATGGTC GGATGACAGG G.AGGGGGAA GAATACAGGA CACCAGCAGA
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 22CiiF  TTTGATGGTC GGATGACAGG G.AGGGGGAA GAATACAGGA CACCAGCAGA
13ciiCF  TTTGATGGTC GGATGACAGG G.AGGGGGAA GAATACAGGA CACCAGCAGA
17ciiCF  TTTGATGGTC GGATGACAGG GCAGGGGGAA GAATACAGGA CACCAGCAGA
 8ciiCF  TTTGATGGTC GGATGACNNG G.AGGGGGAA TAATACACGA CACCAACAGA
   19DF  TTTGATGGTC GGATGACAGG G.AGGGGGAA GAATACACGA CACCACCACA
  17bBF  CNCTCGCTNC CTGCCTCTCT GGCTGTCTGC TNNGGCNGGG CTGCTNGGAT
   3bBF  CCCNCNCNCC CNGCCTCTCT GGNTGTCTGC GGGNGCNGGG CTGCTTNNAT
  24bBF  CNCTCGCTNC CNGCCTCTCT GGCTGTCTGC NNNNGCNGGG CTGCTGGGAT
  14bBF  CCCTCGCTNC CTGCCTCTCT GGCTGTCTGC TNNNGCNGGG CTGCTNGGAT
   20BF  CCCTCGCTGC CTGCCTCTCT GGNTGTCTGC TNTNGCAGGG CTGCTTGNAT
 12diDF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTGGGAT
 13diDF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTGGGAT
  18bBF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TNNNGCNGGG CTGCTGGGAT
  4diDF  CCCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTNGNAT
  7diDF  CCCNCNCTNC CTGCCTCTCT GGCTGTCTGC TTTNGCNGGG CTGCTGGGAT
  1diDF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTGGGAT
  5diDF  CNCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCNGGG CTGCTGGGAT
   22Di  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTGGNAT
  16dDF  CNCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTGGGAT
  23DiF  CCCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTNGNAT
  15dDF  CCCTCGCTNC CTGCCTCTCT GGNTGTCTGC TNTNGCNGGG CTGCTGGNAN
   19BF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGGAGGG CTGCTNGGAN
  19CiF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGGAGGG CTGCTNGGAN
  24dDF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC TTTTGCAGGG CTGCTGGGAT
   2dDF  CGCTCGCTGC CTGCCTCTCT GGCTGTCTGC NTTGGCAGGG CTGCTGGGAT
  8diDF  CCCNCNCNNC CTNCNTNTCT GGNTNTCTGC TANNGCGGGG NTNCNGNNAT
14aiiAF  .......... .......... .......... .......... ..........
18aiiAF  .......... .......... .......... .......... ..........
17aiiAF  .......... .......... .......... .......... ..........
19aiiAF  .......... .......... .......... .......... ..........
 21DiiF  ACCNAGNGGG NNGGGAANCN AAAAANANNA NTCTCTGACC TAGCCGATCT

         151                                                 200
   5cCF  ACTCTATATG CTGCATAGAA CCNGG..ATA GGATTTTTCA NNGTTAATNA
   7cCF  ACTCTATATG CTGCATAGAA CCTGG..ATA GGATTTTTCA NAGTTAATNA
 14aiAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
 17aiAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
 13ciCF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
 19CiiF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
 19aiAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
   1CCF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
  23CiF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
  21CiF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
  8ciCF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA TAGTAAATGA
   2cCF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
   3aAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
   6aAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
 18aiAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
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  24cCF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
  24aAF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
  22CiF  ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTCA AAGTAAATGA
   4cCF  ANTCTATATG CTGCATAAAT CCGNGGATGA GGATTTNTCA GNGNTAATAA
 12ciiCF TTTCTCAACA TTGCTAGCTT GTGTACAATA TANGAAAAGT AGGAGGTGAA
 16ciiCF TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
 15ciiCF TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
 23CiiF  TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
 20CiiF  TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
 22CiiF  TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
 13ciiCF TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
 17ciiCF TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
  8ciiCF TTTCTCANCA TTGCTNGCTT GTGTNCNATA TAGGANCANT AGGACGTGNA
   19DF  NTTCTCACCA TNGCTCGCTT GTGTACAACA TAGGAAAANT ANGANGTGAA
  17bBF  TTTNTNANCT CTGTNANAAT CCNGGGAGTT GGTGATATCA GACTAGTTGG
   3bBF  TTNNTNCGCN CTNNNAGAAN CNGGGGANTT GGTNATATCA GAGTAGTTGG
  24bBF  TTNNNANNCT CTGTGAGAAT CCNGGGAGTT GGTGATATCA NANTAGTTGG
  14bBF  TTTNTAANCT CTGTNANAAT CCNGGGANTT GGTNATATCA GACTAGTNGG
   20BF  TTTNTANNCT CTGTNAGAAT CNNGGGAGTT GGTGATATCA GACTAGTNGG
 12diDF  TTTTTAANCT CTGTNANAAT CCTGGGAGTT GGTGATGTCA GACTAGTTGG
 13diDF  TTTTTAANCT CTGTNANAAT CCTGGGAGTT GGTGATGTCA GACTAGTTGG
  18bBF  TTTNTAAGCT CTGTNANAAT CCNGGGAGTT GGTGATNTCA NACTAGTTGG
  4diDF  TTNTTACGCT CTGTNACAAT CCNGGGANTT GGTGATATCA NACTAGTNGG
  7diDF  TTTNNANNCT CTNTAANAAT CCNGGGANTT GGTGATNTCA AACNAGTNGG
  1diDF  TTTTTAANCT CTGTAANAAT CCTGGGAGTT GGTGATATCA AACTAGTTGG
  5diDF  TTTNTAANCT CTGTNACAAT CNNGGGAGTT GGTGATNTCA GACTAGTNGG
   22Di  TTTTTAANCT CTGTNANAAT CCNGGGAGTT GGTGATATCA NACTAGTTGG
  16dDF  TTTTTNNCCT CTGTNANAAT CCNGGGANTT GGTGATATCA NACTNGTTGG
  23DiF  TTTTTAANCT CTGTAANAAT CCNGGGAGTT GGTGATNTCA NACTAGTTGG
  15dDF  TTTTTANCCT CTGTNAGAAT CCNGGGAGTT GGTNATATCA NACTAGTNGG
   19BF  TT..... ..... ..... ..... .....
  19CiF  TT..... ..... ..... ..... .....
  24dDF  TTTTTAANCT CTGTGAGAAT CCTGGGAGTC GGTGATGTCA GACTAGTTGG
   2dDF  TTTTTAACCT CTGTGANAAT CCTGGGANTT GGTGATGTCA GACTAGTTGG
  8diDF  TTTTTCCNCN CTNTAATAAT CTCGNGANTT NGTNATATNN NANTNNTNGG
 14aiiAF ..... ..... ..... .....GGTAA GTGGCAGAGC
 18aiiAF ..... ..... ..... ...CTGGTAA GTGGCAGAGC
 17aiiAF ..... ..... ..... ...CTGGTAA GTGGCAGAGC
 19aiiAF ..... ..... ..... ....CTGGTA ATGGCAGAGC
 21DiiF  GNTGTGTNCC CACCCACAAC TGGANNANTG TGTNNNNGGA GGTTCTGGTA

         201                                                 250
   5cCF  ATCTCGAAAG CTGGATAGCA TAGCAGACNA GTGCTGTCAA .TTCANCCAG
   7cCF  ATCTCGAAAG CTGGATNGCA TAGCANACNA NTGCTGTCNA .TTCNGCCAG
 14aiAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
 17aiAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
 13ciCF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
 19CiiF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
 19aiAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
   1CCF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
  23CiF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
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  21CiF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
   8ciCF  ATCTCGAATG CTGGATTGCN CAGCANACGA NTGCAGTCNA CTTCAGCNNG
    2cCF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
    3aAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
    6aAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
  18aiAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
   24cCF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
   24aAF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
   22CiF  ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCACTCNA .TTCAGCCAG
    4cCF  ATCTCGAAAG CTGGATAGCA TAGCANACNA ATGCTNTCNA .TTCNNCCAG
 12ciiCF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAN
 16ciiCF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
 15ciiCF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
  23CiiF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
  20CiiF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
  22CiiF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
 13ciiCF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
 17ciiCF  GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
  8ciiCF  CCNGNTGTGG GGTNTGATGA CGTGNACAAN NACCACNCTN NCGGACTGAC
    19DF  CCANNTTTGG GGTATCATCA NCTGGNCCCA CAACANTNTT ACGNACCGC.
   17bBF  GTCNTTTGAA GGTCAGCAGC NNGGGTAGGG TTCACCNANN N.TCCACTCN
    3bBF  GTCNTNNGAA GGTCNNCNGN GGGGGTANGG TNCACCNANC N.TCCACTCN
   24bBF  GTCATTTGAA GGTCANCNNC CNGGGTNNGG TTCACCGANN C.TNCACTCG
   14bBF  GTCATTNNAA GGTCNCCNNC NNGGGTANGG TNCACCNANN N.TCCACTCN
    20BF  GTCATTNNAA GGTCACCNCC NCGGGTAGGG NNCACCAAT. ..........
  12diDF  GTCATTTGAA GGTTAGCAGC CCGGGTAGGG TTCACCGAAA G.TTCACTCN
  13diDF  GTCATTTGAA GGTNACCAGC CCGGGTAGGG TTCACCGAAA G.TTCACTCG
   18bBF  GTCATTTGAA GGTCANCNNC CCGGGTANGG TTCACCNANN N.TCCACTCN
   4diDF  GTCATTNGAA GGTCANCANC CNGGGTNGGG TTCACCGANN N.TNCNCTCG
   7diDF  GTCATNNGAA GGTTNGCNNC CNGGGTNNGG TNCACCNANN N.TNCNCTCT
   1diDF  GTCATTNNAA GGTNACCANC CCGGGTNGGG TNCACCNANN N.TCCNCTCT
   5diDF  GTCATTTGAN GGTCANCNCC CNGGGTAGGG TNCACCTANN C.TNCACTCT
    22Di  GTCATTTGAA GGTTACCAGC CCGGGTAGGG TTCACCGAAA N.TCCACTCG
   16dDF  GTCATTTGAA GGTCANCNGC NNGGGTAGGG TTCACCNANN N.TCCACTCT
   23DiF  GTCATTNNAA GGTCACCANC CNGGGTAGGG TTCACCNANN C.TCCACTCN
   15dDF  GTCANTTGAA NGTCNCCANN CCGGGTANGG TNCACCNAAN N.TCCNCTCT
    19BF  .......... .......... .......... .......... ..........
   19CiF  .......... .......... .......... .......... ..........
   24dDF  GTCATTTGAA GGTTACCAGC CCGGGTAGGG TTCACCGAAA G.TTCACTCG
    2dDF  GTCATTTGAA GGTTAGCACC CCGGGTAGGG TTCACCGAAA GTTTCACTCG
   8diDF  NTCNTTNGNN GGTNACCNGC NNGGNTCNNG GNCCNCCTCN CCTCCCNTCA
 14aiiAF  CATAATTCAA ACTCAGTTGC CTGACTCCAG TGCATGTGCT TTTTCTGGAG
 18aiiAF  CATAATNCAC ACTCANTTGC CTGACNCCAG TGCATGTGCT TTTCCTGGAG
 17aiiAF  CATAATTCAA ACTCAGTTGC CTGACTCCAG TGCATGTGCT TTTTCTGGAG
 19aiiAF  CATAATTCAA ACTCAGTTGC CTGACTCCAG TGCATGTGCT TTTTCTGGAG
  21DiiF  NACANTTANA TNTACANCAC NNAGCNCGNG GGCATGACCT CCCCNCNCGA

          251                                                300
    5cCF  GGGCTTGCTN TAGGGANAAA GAGACCCAAG ACTGTGGAAG ATCCCCCCNN
    7cCF  GGGCTTGCNA TAGGGANAAA GACAACCAAG ACTGTGGAAT GATTCCCCCC
  14aiAF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
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 17aiAF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
 13ciCF  GGGCTTGCAA GAGGGAGAAA GAN.AAAAAG ACTGTGGAAT .GGAAAGTTT
 19CiiF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
 19aiAF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
   1CCF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
  23CiF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
  21CiF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT GGGAAAGTTT
  8ciCF  GGGCTTGCNC NAGGGAGAAA NAN.AACCCG ACTGTG.... ..........
   2cCF  GGGCTTGCAA NAGGGAGAAA GAG.AAAAAG ACTGTGGAAT GGAAAGTTCC
   3aAF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT GGAAAGTTTC
   6aAF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT GGAAAGTTTC
 18aiAF  GGGCTTGCAA GAGGGAGAAA GAGAAAAAAG ACTGTGGAAT GGGAAAGTTT
  24cCF  GGGCTTGCAA GAGGGAGAAA GAG.AAAAAG ACTGTGGAAT .GGAAAGTTT
  24aAF  GGGCTTGCAA GAGGGAGAAA GAG..AAAAG ACTGTGGATG AAGTTCCACC
  22CiF  GGGCTTGCAN GAGGGAGAAA CACAACAAAG ACTGTGGAAT .GGANNGTCC
   4cCF  GGGCTTGCTG TAGGGATAAA GANACCCAAG ACTGTGGAAT GNANTTCCCC
12ciiCF  TGTTTACGTT CCTCCAAAAA TNATATGTT. .......... ..........
16ciiCF  TGTTTACGTT CCTCCAAAAA TTATATGTTG AAATATTAAC CCCCAGTGTG
15ciiCF  TGTTTACGTT CCTCCAAAAA TTATATGTTG AAATATTAAC CCCCAGTGTG
 23CiiF  TGTTTACGTT CCTCCAAAAA TTATATGTGG AAATATTAAC CCCCAGTGTG
 20CiiF  TGTTTACGTT CCTCCAAAAA TTATATGTTG AAATATTAAC CCCCAGTGTG
 22CiiF  TGTTTACGTT CCTCCAAAAA TTATATGTTG AAATATTAAC CCCCAGTGTG
13ciiCF  TGTTTACGTT CCTCCAAAAA TTATATGTTG AAATATTAAC CCCCAGTGTG
17ciiCF  TGTTTACGTT CCTCCAAAAA TTATATGTTG AAATATTAAC CCCCAGTGTG
 8ciiCF  NNNTTACNTA CCCCCAGCNC ATCANATGTG .......... ..........
   19DF  .......... .......... .......... .......... ..........
  17bBF  CATATATN.. ...A.GGCNA TNCANTCTTT CNTTCTGTNT NACAGAATAN
   3bBF  CANATATN.. ...A.NGCNA TNCNNNCTT. .......... ..........
  24bBF  CATATATN.. ...A.NGCNA TCCNATCTTT CTGGCTGTGT NACAGAAGTA
  14bBF  CATATATN.. ...A.GGCNA TCCANTCTTT C......... ..........
   20BF  .......... .......... .......... .......... ..........
 12diDF  CATATATT.. ...A.GGCAA TTCAATCTTT CATTCTGTGT GACAGAAGTA
 13diDF  CATATATT.. ...A.GGCAA TTCAATCTTT CATTCTGTGT GACAAAAGTA
  18bBF  CATATATN.. ...A.GGCAA TNCANNCTTT CNTTCTGTGT GAAATA....
  4diDF  CATATATN.. ...A.GGCAN TCCANTCTTN CATTCTGTGT GACAAAAATA
  7diDF  CATATATN.. ...A.CGCAN TCCANNCTTT CNTTCTGTGT NACAAANNTA
  1diDF  CATATATN.. ...A.GGCAA TNCANTCTTT CNTTCTGTGT NACAAAAGTA
  5diDF  CATATATN.. ...A.NGCNA TNCANTCTTT CATTCTGTGT GACANAANTA
   22Di  CATATATT.. ...A.GGCAA TTCAATCTTT CATTCTGTGT GACAAAAGTA
  16dDF  CATATATT.. ...A.GGCAA TTCANTCTTT CTTTCTGTGT GACAAAAGTA
  23DiF  CATATATN.. ...A.GGCAA TNCAATCTTT CATTCTGTGT NACAAAANTA
  15dDF  CATATATN.. ...A.NGCNA TNCACNCTTT CATTCTGTGT NAAANAAGTA
   19BF  .......... .......... .......... .......... ..........
  19CiF  .......... .......... .......... .......... ..........
  24dDF  CATATATT.. ...A.GGCAA TTCAATCTTT CATTCTGTGT GACAAAACTA
   2dDF  CATATATT.. ...AGGGCAA TTCAATCTTT CATTCTGTGT GACAGAAGTA
  8diDF  CTNTTNNTTT ACGCNTNTCA CNCTCNCTTT AGTCCCGTGN GANGTNACCC
14aiiAF  AGGAGA.... .......... .......... .......... ..........
18aiiAF  AGGAGA.... .......... .......... .......... ..........
17aiiAF  ANAGA..... .......... .......... .......... ..........
19aiiAF  AGAGA..... .......... .......... .......... ..........
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 21DiiF  TNNCCAGCAG ANTTGCAANT CCACTCTCTA CCCCGNGTTG TNNCCTCTCC

         301                                                 350
   5cCF  NNNNNNNCCC CAAGCCCTTT CCCCAAGGGG GTTAGCCCAT TCCTCTGTTC
   7cCF  CCNNNANCCC CAAGCCTTTC CCCAA...GG GGTTAGCCAT TCCTCTGTTC
  14aiAF CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
  17aiAF CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
  13ciCF CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
  19CiiF CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
  19aiAF CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
   1CCF  CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
  23CiF  CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
  21CiF  CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
   8ciCF ..........  ..........  ..........  ..........  ..........
   2cCF  CCCAACCCAA GCCTTTCCCA AGGGG.TAGC CATTCTCTGT TCTACAGTTT
   3aAF  CCAACCCAAG CCTTTCCCAA GGGGT.TAGC CATTCTCTGT TCTACAGTTT
   6aAF  C.......AC CAGCCTTCCA AGGGG.TAGC CATTCTCTGT ..........
  18aiAF CCCAACCCAA GCCTTTCCCA AGGGGTTAGC CATTCTCTGT TCTACAGTTT
  24cCF  CCCAACCCAA GCCTTTCCCA AGGG..TAGC CATTCTCTGT .CTACAGTTT
  24aAF  CAGCCTCCAG GGGTTACCAT ..........  ..........  ..........
  22CiF  CCCNNCCCNA NCCTTTCTCA NGGGGTANCC NNTCCCNGTT TCTNNCTCTT
   4cCF  CCCNNNCCCA AGCCTTTCCC AAGGGGTTNC CATTCTCTGT TCTACAGTTT
 12ciiCF ..........  ..........  ..........  ..........  ..........
 16ciiCF ACAGAAGTAG TAGAAGGAC.  ..........  ..........  ..........
 15ciiCF ACAGAAGTAG TAGGAAGGAC  ..........  ..........  ..........
  23CiiF ACAGAAGTAT GAAG......  ..........  ..........  ..........
  20CiiF ACAGAAGTAG TAGGAA....  ..........  ..........  ..........
  22CiiF ACAGAAGTAT AGAAG.....  ..........  ..........  ..........
 13ciiCF ACAGAAGTAT GAAG......  ..........  ..........  ..........
 17ciiCF ACAGAAGTAT AGAAG.....  ..........  ..........  ..........
  8ciiCF ..........  ..........  ..........  ..........  ..........
   19DF  ..........  ..........  ..........  ..........  ..........
  17bBF  AAGAGA....  ..........  ..........  ..........  ..........
   3bBF  ..........  ..........  ..........  ..........  ..........
  24bBF  GTAGAGGNNN CCCCCGGGAC NCTGCNC...  ..........  ..........
  14bBF  ..........  ..........  ..........  ..........  ..........
   20BF  ..........  ..........  ..........  ..........  ..........
 12diDF  GTAAGAAGTG AGCTGTTCAG AGGCAGGAGG GTCTATTCTT TGCCGGGGGG
 13diDF  GTAAGAAGTG AGCTGTNCAG AGGCAGGAGG GTCTATTCTT TGCNGGGGGG
  18bBF  ..........  ..........  ..........  ..........  ..........
  4diDF  NTATGANNTG ANCTGTTCAN AGGCNGGAGG GTCTANTCTT TG........
  7diDF  TTAAGAANTG ACCTGTNCAN AGGNAGGANG GTCNAC....  ..........
  1diDF  GTAAGAAGTG AGCTGTNCAN AGGCNGGANG GTCTNTTCTT TG........
  5diDF  ATANGAANTG AGCTGTACAG AGGCAGGAGG GTCTNTTCTT  ..........
   22Di  GTAAGAAGTG ANCTGTCCAG AGGCAGGAGG GTCTATTCTT TGNGGGGGGG
  16dDF  NTAAGAANTG AGCTGTNCAN AGGCNGGANG GT........  ..........
  23DiF  NTAAGANGTG ANCTGTTCAN AGGCNGGAGG GTCTNNCCTT TGNGGGGGGG
  15dDF  NTANGAANTN AGCTGTNCAN AGGCNGGAGG GTC.......  ..........
   19BF  ..........  ..........  ..........  ..........  ..........
  19CiF  ..........  ..........  ..........  ..........  ..........
  24dDF  GTANGAAGTG AGCTGTTCAN AGGCNNGAGG GTCTATTCTT TTGCCGNGGG
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   2dDF  GTAAGANNTG AACTGTTCAT AANGGCAGGA AGGTCCNCTT TTGGCCAAAN
   8diDF TGTNGTATTA CGTTTCACTN TACCANTTTT ACCNCCGATT CCACCACTGG
  14aiiAF .......... .......... .......... .......... ..........
  18aiiAF .......... .......... .......... .......... ..........
  17aiiAF .......... .......... .......... .......... ..........
  19aiiAF .......... .......... .......... .......... ..........
   21DiiF NCCCGGNNGG GCAAATGCTC TCNGAAGTGC TACTATNTNT TCACCGAANN

          351                                                  400
    5cCF  CTACAGTTTT AAGGGCTTGG CATGTTGCTT TTTTCCCGGA ATTTGGAAAA
    7cCF  CTACAGTTTT AAGG..CTTG CATGTTGC.T TTTTCCTGGA ATTGGGAAAA
   14aiAF AGGCT.GCTG TCTTC..... .......... .......... ..........
   17aiAF AGGCT.TGTT .......... .......... .......... ..........
   13ciCF AGGGC.TTGC ATGTGCTTTT T.CTGGAGT. GGAAAANTAC ATAAGTTATA
   19CiiF AGGGC.TTGC ATGTGCTTTT T.CTGGAGT. GGAAAAATAC NTAAGTTATA
   19aiAF AGGGC.TTGC TTTCG..... .......... .......... ..........
    1CCF  AGGGC.TTGC ATGTGCTTTT T.CTGGAGT. GGAAAAATAC ATAAGTTATA
   23CiF  AGGGC.TTGC ATGTGCTTTT T.CTGGAGTG GGAAAAATAC ATAAGTTATA
   21CiF  AGGGC.TTGC ATGTGCTTTT C.TGAGTGGG GAAAAAATAC NTAANTATAN
    8ciCF .......... .......... .......... .......... ..........
    2cCF  AGGGCTTTGC ATGTGCTTTT T.CTGGAATT GGAAAAATAC ATAATTTATA
    3aAF  AGGCTT.... .......... .......... .......... ..........
    6aAF  .......... .......... .......... .......... ..........
   18aiAF AAGGCTTGCT .......... .......... .......... ..........
   24cCF  AGGCTTGCAT GTGCTTTTCT G.ATTGGAAA AATACATAGT TATAAGGAAT
   24aAF  .......... .......... .......... .......... ..........
   22CiF  CCAGGGNCTT GCNA..... .......... .......... ..........
    4cCF  AGGGCTTGCA TGTTGCTTTT TCCTGGAGTT GGAAAAATAC ATAAGTTATA
  12ciiCF .......... .......... .......... .......... ..........
  16ciiCF .......... .......... .......... .......... ..........
  15ciiCF .......... .......... .......... .......... ..........
   23CiiF .......... .......... .......... .......... ..........
   20CiiF .......... .......... .......... .......... ..........
   22CiiF .......... .......... .......... .......... ..........
  13ciiCF .......... .......... .......... .......... ..........
  17ciiCF .......... .......... .......... .......... ..........
   8ciiCF .......... .......... .......... .......... ..........
    19DF  .......... .......... .......... .......... ..........
   17bBF  .......... .......... .......... .......... ..........
    3bBF  .......... .......... .......... .......... ..........
   24bBF  .......... .......... .......... .......... ..........
   14bBF  .......... .......... .......... .......... ..........
    20BF  .......... .......... .......... .......... ..........
   12diDF GGGACCACAA TCCCCCCATG CGATCTGTTT GAGGACTGGG ATGCCGAGAA
   13diDF GGGACCACAA TCCCCCCATG CGATCTGTTT GAGGACTGGG ATGCCGAAAA
   18bBF  .......... .......... .......... .......... ..........
   4diDF  .......... .......... .......... .......... ..........
   7diDF  .......... .......... .......... .......... ..........
   1diDF  .......... .......... .......... .......... ..........
   5diDF  .......... .......... .......... .......... ..........
    22Di  GGGANCACAA NCCCCCCATG CNATCTGTTT GAGGACTGGG GATGCCNAAA
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 16dDF  .......... .......... .......... .......... ..........
 23DiF  GGGACCACAN NCCCCCCATG CNATCTGTNN GAGGACTGGG ATNCCNANAA
 15dDF  .......... .......... .......... .......... ..........
  19BF  .......... .......... .......... .......... ..........
 19CiF  .......... .......... .......... .......... ..........
 24dDF  GGGGANCACA ATTCCCCCCA TTCCAACTGT TTTGAGGACN GGGAATCCCC
  2dDF  GGGGGGGAAC AAAAAATNNC CCCCCATGCC AANCTGTTTT GAANAACTGG
 8diDF  GAA....... .......... .......... .......... ..........
4aiiAF  .......... .......... .......... .......... ..........
8aiiAF  .......... .......... .......... .......... ..........
7aiiAF  .......... .......... .......... .......... ..........
9aiiAF  .......... .......... .......... .......... ..........
21Di
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Appendix II

Manuscript submitted

ALL-*TRANS*-RETINOIC ACID MEDIATES G1 ARREST BUT NOT APOPTOSIS OF NORMAL HUMAN MAMMARY EPITHELIAL CELLS [1]

Victoria L. Seewaldt[2], Ji-Hyon Kim, L. Elizabeth Caldwell, Barton S. Johnson, Karen Swisshelm, and Steven J. Collins

Division of Medical Oncology, Department of Medicine, University of Washington, Seattle, Washington 98195 [V.L.S.], Division of Medical Oncology, Department of Medicine, University of Washington, and Division of Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, Washington 98195 [S.J.C.], Program in Electronic Microscopy, Fred Hutchinson Cancer Research Center, Seattle, Washington 98195 [L.E.C.], Division of Hospital Dentistry, Department of Restorative Dentistry, Seattle, Washington 98195 [J.H.K., B.S.J.], Department of Pathology, University of Washington, Seattle, Washington 98195 [K.S.].

Address: Division of Medical Oncology
Box 356460
University of Washington
Seattle, WA 98195

FAX: (206) 543-3519
Phone: (206) 543-3669
email: vseewald@u.washington.edu

ABSTRACT

Retinoids mediate the normal growth of a variety of epithelial cells and may play an important role in the chemoprevention of certain malignancies. Loss of retinoic acid receptor-beta (RARβ) function may be an important event in mammary carcinogenesis since the majority of breast cancers, in contrast with normal mammary epithelial cells, fail to express this receptor. We previously reported that all-*trans*-retinoic acid (RA) mediates G_1 arrest as well as apoptosis in certain RARβ-transduced breast cancer cell lines. We now report the effect of RA on normal human mammary epithelial cells (HMECs), which express functionally active retinoid receptors. We observe that RA induces growth suppression and G_1 arrest of these HMECs but find no evidence that RA mediates apoptosis in these normal cell strains. This RA-induced G_1 arrest is temporally associated with decreased levels of hyperphosphorylated retinoblastoma protein (pRB) without any significant changes in c-myc, p53, p21, or p27 expression. Our studies suggest that growth inhibition, rather than apoptosis, may be the mechanism by which RA and RA receptors act to prevent the malignant transformation of normal mammary epithelial cells. The molecular targets(s) of the activated RA receptors that mediate this G_1 arrest in HMECs appear to be associated with an Rb-dependent pathway.

Running Title: RA-mediated growth inhibition of HMECs

Key words: retinoids, carcinogenesis, cell cycle, mammary epithelial cells, apoptosis, retinoblastoma protein

Footnotes:

[1] This work was supported by grants from the University of Washington Graduate School Fund and a Pilot and Feasibility Award from the Clinical Nutrition Research Unit, NIH DK 35816 [K.S.], US Army Grant 17-94-J4982 [K.S.], a grant from the American Cancer Society, J FRA-534 [K.S.], a Pilot and Feasibility Award from the Clinical Research Unit, NIH NIDDK, 2P30 DK 35816-11 [V.L.S], and NIH grant CA 55397 [S.J.C.]. Victoria L. Seewaldt was supported by NIH K08 Clinical Investigator Award CA 68210-01, a New Investigator Award from the Clinical Nutrition Research Unit NIH NIDDK, 2P30DK 35816-11, and a Susan G. Komen Breast Cancer Fellowship.

[2] To whom requests for reprints should be addressed:

Victoria L. Seewaldt, MD, University of Washington, Department of Medical Oncology, Division of Medical Oncology, Box 356460, Seattle, Washington 98195.

Telephone: (206) 543-3669

Fax: (206) 543-3915

[3] Abbreviations: **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RA**, retinoic acid; **HMEC**, human mammary epithelial cells; **RARβ**, retinoic acid receptor-beta; **RARE**, retinoic acid response element; **pRB**, retinoblastoma protein, **cdk**, cyclin dependent kinase, **TdT**, terminal deoxynucleotidyl transferase; **PBS**, phosphate buffered saline; **ECL**, enhanced chemiluminescent detection; **DAPI**, 4',6-diamido-2-phenylindole; **FACS**, fluorescence activated cell sorting; **RT**, room temperature; **DMSO**, dimethyl sulfoxide; **T.E.**, Tris-ethylenediaminetetraacetic acid; **CAT**, chloramphenicol acetyl transferase.

INTRODUCTION

Vitamin A (retinol) and its derivatives (retinoids) support the normal growth and differentiation of epithelial cells (1, 2). Retinoids are also effective in the prevention of many human malignancies (3-13) and currently there are ongoing clinical trials to test the ability of a synthetic retinoid, N-(4-hydroxyphenyl)retinamide [Fenretinide], to prevent contralateral breast cancer (14, 15). In order to use retinoids in the most clinically beneficial manner, it is important to understand the molecular basis of activity. The actions of retinoids are ultimately thought to be mediated through specific nuclear retinoic acid receptors (RARs)[3] and retinoid X receptors (RXRs) belonging to the steroid/thyroid superfamily of transcription factors (16-20). RARα is expressed ubiquitously in adult tissue and RARγ is primarily expressed in skin. RARβ is unique because it is primarily expressed in epithelial cells and exhibits induced expression in response to retinoic acid mediated by an enhancer element found in its promoter, the retinoic acid response element (RARE) (21, 22).

Since retinoic acid receptors appear to play a key role in mediating retinoid action, it is likely that these receptors also regulate the anticarcinogenic actions of retinoids. This paradigm is consistent with the finding that while RARβ is primarily expressed in many normal epithelial derived-cells or tissues, it is not expressed in a majority of epithelial tumors or in tumor cell lines (23-28). It has been observed that normal human cultured mammary epithelial cells (HMECs) express RARβ mRNA; in comparison, most breast cancer cells fail to express this gene (23, 29, 30). Furthermore, RARβ mRNA expression is upregulated in senescent HMECs (23). This suggests that loss of retinoic acid receptor function may be an important event in solid tumor carcinogenesis.

We previously developed an *in vitro* system to investigate how retinoic acid receptors may

act to prevent the malignant transformation of human mammary epithelial cells (24). The human breast cancer cell lines MCF-7 and MDA-MB-231 do not normally express RARβ and are resistant to RA-mediated growth inhibition at 1.0 μM all-*trans*-retinoic acid (RA). By retroviral mediated gene transfer, we constitutively expressed the human RARβ gene in both of these breast cancer cell lines and demonstrated that RARβ-transduced MCF-7 and MDA-MB-231 cells readily undergo growth inhibition when treated with RA (24). In addition, the RARβ-transduced MCF-7 cells undergo apoptosis after 4 days of treatment with RA (24). This suggests that RA and RARβ are important mediators of proliferation in these breast cancer cell lines and provides insight into how RA might act to prevent cancer in normal epithelial cells. If the mechanism of action of retinoids and retinoic acid receptors in normal human mammary epithelial cells is to regulate proliferation and apoptosis, then loss of retinoid receptor function might disrupt an important mechanism of maintaining normal tissue homeostasis and thereby contribute to malignant transformation.

In this report we investigate the growth regulation of normal mammary epithelial cells (HMECs) by retinoic acid. HMECs are growth factor-dependent cells derived from reduction mammoplasty specimens and exhibit a limited *in vitro* lifespan. We observe that RA-treated HMECs in culture undergo growth inhibition predominantly associated with G_1 arrest but they do not undergo apoptosis. While p53, p21, and c-myc levels remain constant in HMECs which have undergone this RA-mediated G_1 arrest, there is a marked reduction in the levels of hyperphosphorylated pRB. This is consistent with previous observations in RA-treated T-47D breast cancer cells (31). Our results suggest that retinoids and retinoic acid receptors may be important mediators of proliferation in HMECs but do not play an important role in mediating apoptosis in these cells.

RESULTS

NORMAL HUMAN MAMMARY EPITHELIAL CELLS (HMECS) EXPRESS RA-INDUCIBLE RARβ MRNA

We determined the levels of retinoic acid receptor-α (RARα) and retinoic acid receptor-β (RARβ) mRNA in normal human mammary epithelial cell strains AG11132 and AG11134 with and without treatment with 1 μM RA for 48 hours. The expected 3.6 and 2.8 kb RARα messages were observed in both RA-treated and untreated HMECs. AG11132 RARα mRNA levels were slightly increased by treatment with RA. In contrast, RARα mRNA levels increased three fold in AG11134 after RA-treatment (Figure 1). Unlike most breast cancer cell lines (23, 24), HMEC strains AG11132 and AG11134 express RARβ mRNA, albeit at low levels relative to RARα. RARβ mRNA levels increased 2- and 4- fold respectively for AG11132 and AG11134 after treatment with 1 μM RA (Figure 1). These HMEC strains also expressed RXRβ (at levels comparable to RARα) and low levels of RARγ mRNA (data not shown).

RA MEDIATED *TRANS*-ACTIVATION IN CULTURED HMECS

Since HMEC strains exhibit RA-inducible RARβ expression (Figure 1), we wished to determine whether the retinoid receptors expressed in these HMEC strains are functional and able to *trans*-activate a retinoic acid response element in response to RA. We performed transient expression assays utilizing the pRRE4-tkCAT reporter plasmid which contains the natural RA-response element (RARE) from the promotor region of the human RARβ gene (22). Retinoid receptor functional activity was demonstrated in HMEC strains AG11132 and AG11134 by induction of RA-mediated *trans*-activation with increasing

concentrations of RA (Figure 2).

GROWTH INHIBITION AND CELL CYCLE CHANGES OBSERVED IN NORMAL HUMAN MAMMARY EPITHELIAL CELL STRAINS IN RESPONSE TO RETINOIC ACID

HMEC strains AG11132 and AG11134 were cultured in 0, 0.1, 1.0, or 10 μM RA. We observed RA-mediated growth inhibition of HMECs that was both dose and time dependent. Increased growth inhibition was demonstrated with increasing concentrations of RA and increasing time of exposure in both AG11132 and AG11134 (Figure 3). Growth inhibition after treatment with 1.0 μM RA was observed starting at 24 hours. Ten micromolar RA appeared cytotoxic to HMECs. These data demonstrate that RA inhibits the proliferation of normal mammary epithelial cells in culture.

In order to determine more precisely the mechanism by which retinoic acid inhibits the proliferation of HMECs, FACS analysis was performed on AG11132 and AG11134 cells treated with 0, 0.1, and 1.0 μM RA for 48 hours. HMECs treated with 0.1 or 1.0 μM RA for 48 hours exhibited an increase in the percentage of cells in G_1 and a decrease in the percentage of cells in S-phase relative to untreated controls (Table 1). After 48 hour of treatment with 1.0 μM RA, AG11132 and AG11134 cells, respectively, exhibit a 17% and 13% increase in G_1 and a 58% and 68% decrease in S-phase. These results demonstrate that RA may inhibit proliferation of HMECs primarily by inducing G_1 arrest.

The effects of 1.0 μM RA on HMEC cell cycle phase distribution were examined over a defined time course to investigate the kinetics of RA-mediated G_1 arrest. Figures 4 A and B shows that both AG11132 and AG11134 cell strains undergo growth inhibition starting 24 hours after treatment with RA. After 48 hours both cell strains exhibited a greater than

50% reduction in the percentage of cells in S-phase and a corresponding increase in the number of cells in G_1.

ALL-*TRANS*-RETINOIC ACID DOES NOT INDUCE APOPTOSIS IN RA-TREATED HMECS

We previously reported that MCF-7 cells transduced with RARβ underwent apoptosis when treated with RA (24). In contrast, MDA-MB-231 cells transduced with RARβ underwent G_1 arrest but did not undergo apoptosis when treated with RA (24). In order to determine whether RA mediates apoptosis in normal human mammary epithelial cells, we treated HMEC strains AG11132 and AG11134 with 1 μM all-*trans*-retinoic acid and investigated whether apoptosis was observed by morphologic criteria and by biochemical parameters.

Morphologic changes characteristic of apoptosis include nuclear condensation, loss of adherence, and cell shrinkage (33, 34). HMECs treated with 1 μM RA did not exhibit these morphologic changes by either light or electron microscopy (data not shown). HMECs treated with 1 μM RA for 5 days did not demonstrate nuclear condensation by fluorescent staining (Figure 5).

Internucleosomal DNA fragmentation is characteristic of apoptosis and distinguishes it from other modes of cell death such as necrosis (35, 36). MCF7-RARβ transduced cells undergoing RA-mediated apoptosis demonstrate increased DNA fragmentation starting 4 days after treatment with RA (24). In contrast, we did not observe increased fragmented cytoplasmic DNA by the diphenylamine assay 2, 4, or 6 days after treatment of HMECs with 1 μM RA (Figure 6). Moreover, ethidium bromide-stained DNA extracted from RA-treated HMECs did not demonstrate DNA laddering following two, four, or six days

treatment with 1 μM RA (data not shown). Finally, apoptotic strand breaks were not detected by the terminal deoxynucleotidyl transferase (TdT) method in HMECs after 5 days treatment with 1 μM RA (data not shown). These together data suggest that while RA induces growth arrest of two independent normal mammary epithelial strains in culture, RA does not induce apoptosis in either of these cell strains.

RA-TREATED HMECS DEMONSTRATE A REDUCTION IN THE LEVELS OF HYPERPHOSPHORYLATED pRB

We performed Northern and Western analysis to determine whether the RA-mediated growth arrest observed in RA-treated HMECs was associated with a change in expression of specific genes important in growth control and proliferation of epithelial cells, including c-myc, p53, p21, p27, and pRB (37-56).

The c-myc proto-oncogene is a transcription factor that participates in the opposing cellular fates of proliferation and apoptosis. We investigated the expression of c-myc mRNA expression in normal mammary epithelial cells strains AG11132 and AG11134 which do undergo G_1 arrest and observed that neither HMEC cell strain demonstrates a change in c-myc mRNA expression after 48 of treatment with RA (Figure 7).

The tumor suppressor protein pRB appears to play a critical role in mediating cell cycle progression (48-56). We observe that RA-mediated growth inhibition in HMEC strains AG11132 and AG11134 is temporally associated with a significant decrease in the level of hyperphosphorylated pRB (Figure 8 and 9). We observe that both HMEC strains undergo G_1 arrest starting at 24 hours following retinoic acid treatment (Figure 4). Concomitant with this observed growth arrest, the levels of hyperphosphorylated pRB begin to decline

after 24 hours of treatment with RA (Figure 9). After 48 hours the levels of hyperphosphorylated pRB are reduced by 50% and 63% for AG11132 and AG11134, respectively (Figure 9). These observations suggest that inhibition of pRB hyperphosphorylation is temporally associated with the RA-induced G_1 arrest in HMECs.

To determine whether this RA-induced hypophosphorylation of pRB was associated with any of the known inhibitors of cyclin dependent kinase activity, we compared the relative expression of p53, p21/WAF1/CIP1, and p27 in untreated and RA-treated HMEC strains. We observe that AG11132 and AG11134 HMEC cells strains treated with 1.0 μM RA for 24 hours (data not shown) or 48 hours (Figure 8) do not exhibit increased p53 or p21 protein levels associated with this RA-induced G_1/S arrest. Both AG11132 and AG11134 HMEC strains express low levels of p27 mRNA (Figure 7), but neither cell strain express detectable levels of p27 protein before or after RA-treatment for 24 or 48 hours (data not shown).

DISCUSSION

The association between vitamin A deficiency and the development of cancer suggests that retinoid-dependent signaling pathways have a role in the suppression of carcinogenesis (2). Therefore, for the effective clinical use of retinoids, it is important to define how retinoid action is mediated in both malignant and normal mammary epithelial cells. The retinoic acid receptor-beta (RARβ) is primarily expressed in epithelial cells and appears to be a critical mediator of retinoid action (23, 24). While RARβ is expressed in normal epithelial tissues, its expression is selectively lost in many epithelial tumors suggesting that the loss of RARβ expression may be a key event in solid tumor carcinogenesis (23-28). It has recently been reported that RARβ expression is selectively lost in patients with oral dysplasia and that a

clinical response to 13-*cis*-retinoic acid was associated with restoration of RARβ expression (57).

In previous studies, we investigated how RA and RARβ might act in breast cancer cells by utilizing retroviral-mediated gene transfer to introduce RARβ into breast cancer cell lines which do not normally express RARβ (24). We observed that while MCF-7 and MDA-MB-231 breast cancer cell lines are resistant to RA-mediated growth inhibition, RA induces G_1 arrest in these two breast cancer cell lines when they are transduced with RARβ. Moreover, RA induces apoptosis in RARβ-transduced MCF-7 cells but not in RARβ-transduced MDA-MB-231 (24). A recent report by Liu *et al.* (58) supports these findings by demonstrating that RARβ mediates the growth inhibitory effects of retinoic acid by promoting apoptosis in human breast cancer cell lines. Furthermore, there is evidence that retinoids and RARβ may mediate apoptosis in other cell types since RA-mediated truncation defects of the embryonic limb appear to be the result of RARβ-mediated apoptosis (59). Based on such observations we hypothesized that retinoids and the retinoic acid receptors could participate in G_1 arrest and apoptosis in normal human mammary epithelial cells.

The normal mammary epithelial cell strains, AG11132 and AG11134, described in this study are derived from reduction mammoplasty specimens and consist of a heterogeneous population of growth factor dependent epithelial-derived mammary cells. Unlike immortal cell lines which retain characteristics of normal cells such as MCF-10A (60), HMEC strains AG11132 and AG11134 are not immortal. In addition, while most breast cancer cell lines do not express RARβ, these HMEC strains express RA-inducible RARβ mRNA (Figure 1). Moreover, the retinoid receptors in these HMEC strains demonstrate normal functional activity as evidenced by RA-mediated *trans*-activation of a RARE driven CAT reporter construct (Figure 2). We wished to determine whether RA might induce growth arrest and/or apoptosis of these HMECs. We observe that RA inhibits proliferation of HMECs

and this is associated primarily with G_1 arrest (Table 1, Figure 4) rather than apoptosis (Figures 5 and 6). In contrast to previous observations in RARβ-transduced breast cancer cell lines (24, 58), we do not observe that RA mediates apoptosis in these two HMEC strains. The absence of apoptosis is confirmed by the lack of characteristic morphologic changes by both light and electron microscopy, and the absence of fragmented DNA (Figures 5 and 6). These data indicate that RA and retinoid receptors mediate G_1 arrest but not apoptosis in normal human mammary epithelial cells.

The observations described in this report may provide a potential model for how RA may act to prevent malignant transformation of normal mammary epithelial cells. If a critical function of RA and retinoic acid receptors is to regulate proliferation, then loss of retinoid receptor function might result in dysregulated growth of human mammary epithelial cells. Further mutations of tumor suppressor genes or oncogenes in this expanded cell population could result in the progression to overt malignancy. This model predicts that loss of retinoid receptor expression and/or function is likely to be an early event in the multistage development of breast cancer in normal mammary epithelial cells.

Growth factors are thought to regulate transit from G_1 to S-phase in almost all cell types but the specific growth factor requirements of mammary epithelial cells are only partially understood. In this report we provide evidence that the differentiating agent RA may play an important role in regulating the G_1 to S-phase cell cycle transition in normal human mammary epithelial cells. Our data suggest that G_1 arrest in HMECs is temporally associated with a decrease in the levels of hyperphosphorylated pRB (Figure 8) in the absence of change in the expression of p53, p21, or p27 protein (Figures 7 and 8) or the expression of c-myc mRNA (Figure 7).

The c-myc proto-oncogene is a transcription factor that participates in the opposing cellular

fates of proliferation and apoptosis. We previously investigated the expression of c-myc mRNA expression in RA-treated RARβ transduced breast cancer cell lines and vector controls and observed that the RA-mediated growth arrest of MDA231-RARβ transduced cells is associated with c-myc down regulation relative to controls (after 48 hours treatment) whereas, the RA-induced apoptosis of MCF7-RARβ transduced cells is not associated with c-myc down regulation (24). From these observations we hypothesized that the lack of c-myc down regulation noted in the RA-treated MCF7-RARβ transduced cells might be related to their ability to undergo RA-mediated apoptosis. We likewise investigated the expression of c-myc mRNA expression in normal mammary epithelial cells strains AG11132 and AG11134 which do undergo G_1 arrest but not apoptosis. Unlike MDA-MB-231-RARβ transduced cells, we observe that neither HMEC cell strain demonstrates a decrease in c-myc mRNA expression after 48 of treatment with RA (Figure 7).

The tumor suppressor protein pRB appears to play a critical role in mediating cell cycle progression. The molecular basis cell cycle progression through the late G_1 restriction point is not fully understood but it appears to correlate with the hyperphosphorylation of pRB. pRB is underphosphorylated throughout G_1 phase, phosphorylated just prior to cells entering S-phase, and remains phosphorylated until late mitosis (48-55). Mutations in Rb are implicated in cell cycle dysregulation and appear to be important in the genesis of a wide variety of cancers (56). We observe that RA-mediated growth inhibition in HMEC strains AG11132 and AG11134 is temporally associated with a significant decrease in the level of hyperphosphorylated pRB (Figure 8 and 9). We observe that both HMEC strains undergo G_1 arrest starting at 24 hours following retinoic acid treatment (Figure 4). Concomitant with this observed growth arrest, the levels of hyperphosphorylated pRB begin to decline after 24 hours of treatment with RA (Figure 9) indicating that inhibition of pRB phosphorylation is temporally associated with RA-induced G_1 arrest in HMECs.

Radiation and other DNA-damaging agents have been found to block pRB hyperphosphorylation. The cell cycle checkpoint gene p53 appears to play an important role in mediating G_1 arrest induced by these agents (37,38). Current models suggest that when cells are exposed to DNA damaging agents such as radiation or chemotherapy, p53 expression is induced. Elevated p53 levels in turn lead to induction of the cyclin inhibitor p21/WAF1/CIP1 resulting in G_1/S arrest associated with pRB hypophosphorylation (39-44). The mechanism(s) by which differentiating agents such as RA might mediate G_1/S arrest may be different than those observed for DNA-damaging agents. We did not observe any increases in either p53 or p21 protein expression when AG11132 and AG11134 HMEC cell strains were treated with RA (Figure 8). Thus the G_1/S growth arrest noted in RA-treated HMECs may occur through a p53 and p21-independent pathway.

The potential link between retinoids and pRB may provide a clue as to how loss of retinoid receptor function in normal mammary epithelial cells might promote breast cancer carcinogenesis. pRB is felt to regulate cell cycle proliferation by association/dissociation with transcription factors, such as E2F (61). Recent data suggests that pRB may regulate more aspects of the cell cycle than previously suspected. Data presented by Cavanaugh *et al.* (62), suggest that pRB might regulate ribosomal RNA transcription mediated by Pol I. This model, that pRB might be a more general regulator of transcription needed for cell proliferation is supported by a recent report by White *et al.* (63), demonstrating that pRB represses a majority of genes transcribed by Pol III in cultured primary mouse fibroblasts. It is currently not known whether pRB hyperphosphorylation in late G_1 causes an increase in Pol-III-mediated transcription.

The question remains whether the observed decrease in hyperphosphorylated pRB is mediated though inhibition of cyclin/cdk complexes or is regulated through other

mechanisms. We are currently investigating the expression of specific cyclins and their respective cyclin dependent kinases, such as cyclin D1/cdk4 and cyclin E/cdk2, felt to be important in phosphorylation of pRB. In a previous report by Wilcken *et al.* (31), RA-treatment did not have an effect on the protein levels of cyclins D1 or E and cdk2 or cdk4 in the breast cancer cell line T47-D. Furthermore, while decreases were observed in cdk2 and cdk4 kinase activity in RA-treated T47-D cells, these changes were observed only after a decrease in the level of pRB phosphorylation occurred (31). These observations, as well as those presented here, suggest that growth regulation by the differentiating agent, RA may be manifest though a pRB-dependent pathway but the precise mechanism remains to be elucidated.

MATERIALS AND METHODS

MATERIALS

All-*trans*-retinoic acid (Sigma) 1 mM stock solution was prepared in 100% ethanol and stored in opaque tubes at -70°C. Control cultures received equivalent volumes of ethanol. Retinoic acid stocks were used under reduced light.

CELL LINES AND MEDIA

Normal human mammary epithelial cell (HMEC) strains AG11132 (M. Stampfer #172R/AA7) and AG11134 (M. Stampfer #48R/AC170) were purchased from the National Institute of Aging, Aging Cell Culture Repository (Coriell Institute) (32). AG11132 and AG11134 strains are cultivated from normal tissue obtained at reduction mammoplasty. These normal cell strains have a limited life span in culture and fail to divide after approximately 20 passages. AG11132 was at passage 8 and AG11134 was at passage 6 at the time of receipt. HMECs are grown in Mammary Epithelial Cell Basal Medium (Clonetics) supplemented with bovine pituitary extract (Clonetics #CC4009) 4 µl/ml, insulin (UBI) 5 µg/ml, epidermal growth factor (UBI) 10 ng/ml, hydrocortisone (Sigma) 0.5 µg/ml, isoproterenol (Sigma) 10 µM, HEPES buffer (Sigma) 10 mM [standard medium]. Cells were cultured at 37° C in a humidified incubator with 5% CO_2/95% air. We did not process our growth media to remove endogenous retinoids. Mycoplasma testing was performed as previously reported by Russell (64).

NORTHERN BLOTTING

RNA was extracted with guanidine isothiocyanate and subjected to Northern blotting in formaldehyde denaturing gels as previously described (23). Ten micrograms of RNA were loaded per lane. Molecular probes utilized in the Northern analysis are as follows: The

hRARα probe is a 1.3 kb, SmaI fragment (17). The hRARβ probe is a 1.4 kb SacI, BamHI fragment (17). The human c-myc probe is a 1.8-kb EcoRI fragment (24). The p27 probe is a 900 bp fragment (gift of Jim Roberts). The 36B4 probe (700bp PstI fragment) (65) was used as a loading and transfer control probe.

CAT REPORTER ASSAY

HMECs were plated in T-75 tissue culture flasks (Corning) in standard media 24 hours prior to transfection and were approximately 50% confluent at the time of transfection. Cells were transfected by CellFECTIN^{tm} (Gibco/BRL Life Technologies) as per the manufacturer's recommendations for transient transfection of adherent cells. Transient transfections were performed utilizing the pRRE4-tkCAT reporter plasmid which contains four copies of the natural RA-response element (RARE) present in the promotor region of the human RARβ gene (22). Transfection control was provided by the pCMV-GH plasmid (34). Transfection conditions were as follows: 10 μl CellFECTIN^{tm} in 1.0 ml standard medium was added to 1.0 ml of standard medium containing 10 μg of pRRE4-tkCAT reporter plasmid (24) and 10 μg of pCMV-GH (32), incubated for 10 mins at room temperature, and then added per T-75 flask. Twenty-four hours after transfection, the cells were washed with PBS and re-fed with standard medium containing 0, 0.1, 1.0, or 10 μM RA. Control cultures received an equivalent volume of ethanol (0.1%). After 24 hours, culture media was collected for determination of growth hormone concentration using a radioimmunoassay kit (Nichols Institute). Preparation of cell lysates and CAT assays were performed according to published methods (66). Twenty microliters of cellular extract was added to 80 microliters of CAT reaction mixture [final concentration: 0.1 M Tris pH 7.8, 2.0 nM n-butyrl Coenzyme A (Sigma), 0.15 μCi [^{14}C]-labeled chloramphenicol (Amersham)] and incubated for 3 hours at 37° C. The reaction mixture was extracted with 200 μl xylenes, vortexed 30 seconds, and centrifuged at 4,000 rpm for 5 min. One hundred seventy microliters of the upper phase was transferred to a new Eppendorf tube

and extracted with 85 µl T.E. [10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid]. One hundred forty microliters of the upper phase was transferred to a scintillation vial and counted. Protein concentration of cell lysates were determined by a Pierce bicinchoninic acid commercial kit. CAT counts were normalized for transfection efficiency as determined by the growth hormone reporter internal control and total protein. Controls were performed to insure that the pCMV-GH plasmid did not demonstrate increased growth hormone activity in response to retinoic acid.

CELL GROWTH CURVES

HMECs were plated in duplicate at $1x10^4$ per well in 24 well plates (Corning) and grown in standard medium with 0, 0.1, or 1.0 µM RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were trypsinized at 12 to 24 hour time intervals and counted in triplicate. Cultures did not exceed ~70% confluency.

DNA STAINING OF CELL NUCLEI WITH PROPIDIUM IODIDE AND FACS SCAN ANALYSIS

$5x10^5$ cells were plated in T75 flasks (Corning) and grown in standard medium with 0, 0.1, 1.0, or 10 µM RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were harvested at 2 days and did not exceed ~70% confluency. For the RA time course: $5x10^5$ cells were plated in T75 flasks on day -1 and allowed to adhere. On day 0 the medium was removed and replaced by 20 ml of fresh medium. RA was added to the culture medium for a final concentration of 1 µM on days 0, 1, 2, and 3 for the preparation of day 4, 3, 2, and 1 time points respectively. Cells were harvested on day 4 and did not exceed ~70% confluency. Preparation of cells for FACS analysis has been previously described (24). Ten thousand events were collected in list mode fashion, stored, and analyzed on Multicycle AV software (Phoenix Flow Systems).

NUCLEAR STAINING OF RA-TREATED HMECS

$1x10^5$ cells were plated in T75 flasks (Corning) and grown in standard medium with or without 1.0 μM RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were harvested at 5 days and did not exceed ~70% confluency, were trypsinized and washed once in cold PBS (pH 7.4), and were fixed in 1% formaldehyde in PBS for 15 mins on ice and washed once with cold PBS. Cells were resuspended in 70% ice cold ethanol and immediately transferred to -20° C. Cytospin preparations utilized $1x10^4$ prepared cells, spun at 400 rpm for 10 mins in a Cytospin 3 centrifuge (Shandon). Cytospin preparations were stained with 4',6-diamido-2-phenylindole (DAPI) solution [10 μg/ml DAPI in 10% dimethyl sulfoxide (DMSO), 0.85% NaCl, 10 mM Tris (pH 7.4), 5 mM $CaCl_2$, 20 mM $MgCl_2$, and 0.5% bovine serum albumin] for 10 minutes and then washed three times with PBS. Fluorescence was analyzed utilizing a Axiophot microscope (Zeiss).

ELECTRON MICROSCOPY

1 x 10^4 cells were plated per well in 6 well plates (Corning) and allowed to attach in standard medium. Retinoic acid stock was added after 24 hours for a final concentration of 1 μM. After 5 days exposure to RA, cells were then fixed in half-strength Karnovsky's fixative (67) for 6 hours, rinsed in 0.1 M sodium cacodylate buffer and post-fixed in 1% collidine-buffered osmium tetroxide. Dehydration in graded ethanol and propylene oxide was followed by infiltration and embedding in Epon 812. Approximately 70-90 nm sections were stained using saturated aqueous uranyl acetate and lead tartrate. Photographs were taken using a JEOL 100 SX transmission electron microscope operating at 80 kV.

DIPHENYLAMINE ASSAY FOR DETERMINATION OF CYTOPLASMIC DNA FRACTION

2.5 to 5.0 x 10^5 cells were plated per T25 flask (Corning) and grown in standard media. Retinoic acid stock was added on days 0, 2, and 4, directly to the media to bring the final concentration to 1 μM. Confluency did not exceed ~70%. On day 6 cells were trypsinized

and washed in cold PBS. The diphenylamine assay was performed as previously described (24). Tubes containing nuclear and cytoplasmic fractions were incubated 16 to 20 hours at 30° C. Optical density was read at 600 nm.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE ASSAY

Cytospin preparations were made in the same manner as described above for DAPI staining. Each slide preparation received 50 μL of TdT buffer (0.1 M sodium cacodylate (pH 7.0) (Sigma), 1 mM calcium chloride (Sigma), 0.1 mM dithiothreitol (Sigma), 0.05 mg/mL bovine serum albumin, 10 units terminal transferase (Boehringer Mannheim) and 0.5 nM fluorescein-16-dUTP (Boehringer Mannheim) (24). Cells were incubated in this solution for 1 hour at 30° C in the dark. Slides were then rinsed three times in PBS. Fluorescence was visualized using a Axiophot microscope (Zeiss).

WESTERN BLOTTING

Preparation of cellular lysates and immunoblotting are as previously described (24). Equal amounts of protein lysates (approximately 100 μg total protein) were loaded on 6%, 10%, or 15% polyacrylamide gels and then electroblotted (Hoeffer) at 80 mA for 45 mins onto Hybond-ECL membrane (Amersham). The membrane was blocked with 20% bovine serum albumin (Sigma) in PBS overnight at RT and then incubated with a 1:100 dilution of either mouse anti-human p53 (Oncogene Science Ab-2), mouse anti-human p21/WAF1 antibody (Oncogene Science Ab-1), or mouse anti-human pRB antibody (PharMingen) for one hour at RT with agitation. The membrane was washed three to five times at RT with 250 mL PBS containing 0.1% TWEEN and then incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) at a 1:35,000 dilution for 1 at RT. The blot was washed again and complexes detected by using ECL Western Blotting Detection Reagents (Amersham) as described by the manufacturer. Detection of p27 protein was detected with a polyclonal rabbit antiserum to p27 protein at a 1:1000 dilution

(gift of Jim S. Robert), washed as described above, and detected with horseradish peroxidase-conjugated Protein A (Sigma) at a 1:2000 dilution and detected using ECL Western Blotting Detection system.

ACKNOWLEDGMENTS

The authors wish to acknowledge the generous gift of p27 antiserum and the p27 molecular probe from Dr. Jim S. Roberts. We also acknowledge the superb technical assistance of LeMoyne Mueller and the excellent editing assistance of Laurel Feltz.

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Table 1: Effects of retinoic acid on HMEC cell cycle

Cells/Retinoic Acid	% G_0/G_1	% change	% S	% change	% G_2/M
AG11132					
No Retinoic Acid	72	--	21	--	7
0.1 μM RA	76	+6	12	-43	11
1.0 μM RA	84	+17	9	-58	7
AG11134					
No Retinoic Acid	71	--	19	--	11
0.1 μM RA	79	+11	11	-42	11
1.0 μM RA	80	+13	6	-68	13

HMEC strains AG11132 (passage 14) and AG11134 (passage 15) were treated with 1.0 μM RA for 2 days. The distribution of cells in the various phases of the cell cycle was determined by flow cytometry as described in Materials and Methods. These data are representative of three separate experiments.

Figure 1: Expression of RARα and RARβ mRNA in RA-treated HMECs.

Northern analysis of HMEC strains AG11132 (passage 12) and AG11134 (passage 10) treated for 48 hours with (+) and without (-) 1.0 μM RA, demonstrating the expression of the RARα and RARβ mRNA. 10 μg of RNA were loaded per lane. Autoradiographic exposure times are 4 and 14 days for RARα and RARβ, respectively. 36B4 is a 1.5 kb mRNA expressed uniformly in all breast cell strains or lines and, therefore, serves as a loading control (64).

Figure 2: RA-mediated *trans*-activation of βRARE in HMECs.

CAT reporter assays of RA-treated HMEC strains AG11132 (passage 11 to 14) (**A**) and AG11134 (passage 9 to 12) (**B**) transfected with pRRE4-tkCAT plasmid. Cells were treated with 0, 0.1, 1.0, and 10 μM RA for 24 hours. pCMV-GH was used as a transfection control. CAT counts were corrected for growth hormone activity and total protein (see Materials and Methods). Data represent the mean of three independent transfections performed in duplicate.

Figure 3: RA-mediated growth inhibition of HMECs.

Growth curves of HMEC strains AG11132 (passage 12) (**A**) and AG11134 (passage 10) (**B**). Cells were plated on Day -1 in standard medium in duplicate at $1x10^4$ cells per well. Cells were re-fed on Day 0 with standard medium containing 0, 0.1, or 1.0 μM RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were trypsinized at 12 to 24 hour time intervals and counted in triplicate.

Figure 4: Effect of RA on cell cycle phase distribution in HMECs.

Cell cycle distribution of HMEC strains AG11132 (passage 14) (**A**) and AG11134 (passage 14) (**B**) treated with 1 μM RA. Cells were plated on Day -1 in standard medium, re-fed on day 0, and treated with 1 mM RA on days 0, 1, 2, and 3. Cells were harvested on day 4. (See Materials and Methods.) Data are presented relative to the %S and $\%G_1$ phase of the untreated cells and are representative of 3 separate experiments.

Figure 5: Nuclear staining of RA-treated HMECs.

HMEC strains AG11132 (passage 13) (**A,C**) and AG11134 (passage 11) (**B,D**) were incubated with (**C,D**) and without (**A,B**) 1.0 μM RA acid for 5 days and then stained with DAPI as described in Materials and Methods. HMECs treated with RA did not exhibit nuclear condensation characteristic of apoptosis.

Figure 6: Cytoplasmic DNA fraction in RA treated HMECs.

The indicated cells were treated for 0, 2, 4, and 6 days with 1.0 μM RA. Cytoplasmic DNA was determined by the diphenylamine assay as described in Materials and Methods. HMEC strains AG11132 (passage 12) and AG11134 (passage 10) do not demonstrate cytoplasmic fragmented DNA. HL-60 cells exhibit retinoic acid-induced apoptosis (68) and serve as a positive control.

Figure 7: Expression of c-myc and p27 mRNA in RA-treated HMECs.

Northern analysis of HMEC strains AG11132 (passage 12) and AG11134 (passage 10) treated for 48 hours with (+) and without (-) 1.0 μM RA, demonstrating the expression of the c-myc and p27 mRNA. 10 μg of RNA were loaded per lane. Exposure time was 3 days and 7 days respectively for c-myc and p27. 36B4 is a 1.5 kb mRNA expressed uniformly in all breast cell lines and therefore serves as a loading control (64).

Figure 8: Expression of pRB, p53, and p21 protein following treatment with all-*trans*-retinoic acid.

AG11132 (passage 11) and AG11134 (passage 12) treated for 48 hours with (+) and without (-) 1 μM RA and analyzed for pRB, p53, and p21 protein expression. Equal amounts of protein lysate were loaded per lane. Incubation was with a 1:100 dilution of anti-pRB antibody (PharMingen), a 1:100 dilution p53 specific antibody (Oncogene Science), or a 1:100 dilution of p21 specific antibody (Oncogene Science) and detected by chemiluminescence as described in Materials and Methods. The arrow denotes the location of the hyperphosphorylated pRB protein. The protein gel was stained with Coomassie blue and an unidentified 45 kd protein was used as a loading control.

Figure 9: Relative expression of hyperphosphorylated pRB following treatment with all-*trans*-retinoic acid.

(**A**) AG11132 (passage 10) and (**B**) AG11134 (passage 8) were treated for 0, 24, and 48 hours with 1 μM RA and analyzed for relative levels of hyperphosphorylated and phosphorylated pRB protein expression. Equal amounts of protein lysate were loaded per lane. Levels of pRB were detected utilizing a 1:100 dilution of antibody to pRB (PharMingen) and detected with chemiluminescence (Amersham). Densitometer readings were made from resulting autoradiograph. Readings were standardized relative to an unidentified 45 kd protein detected by Coomasie blue staining of the original gel.

Figure 1

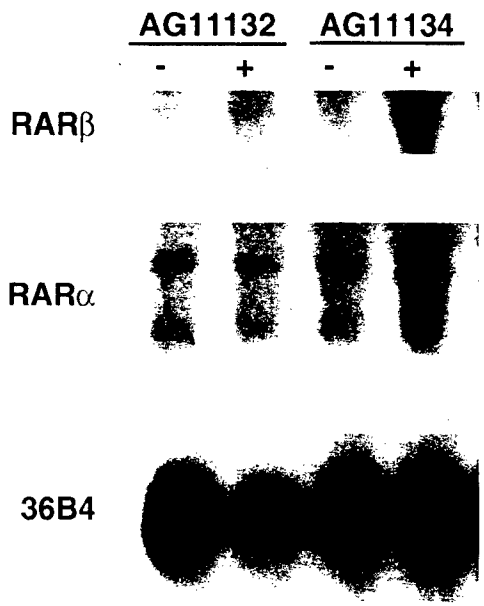


Figure 2

A AG11132

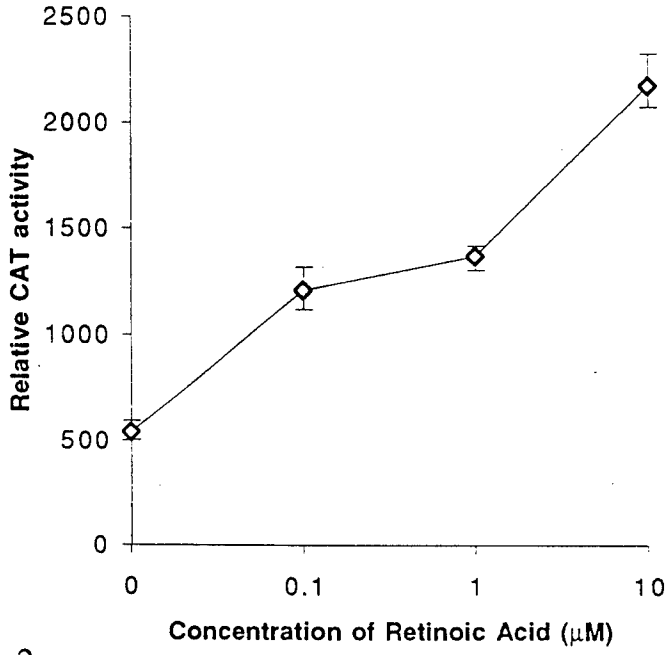


Figure 2

B AG11134

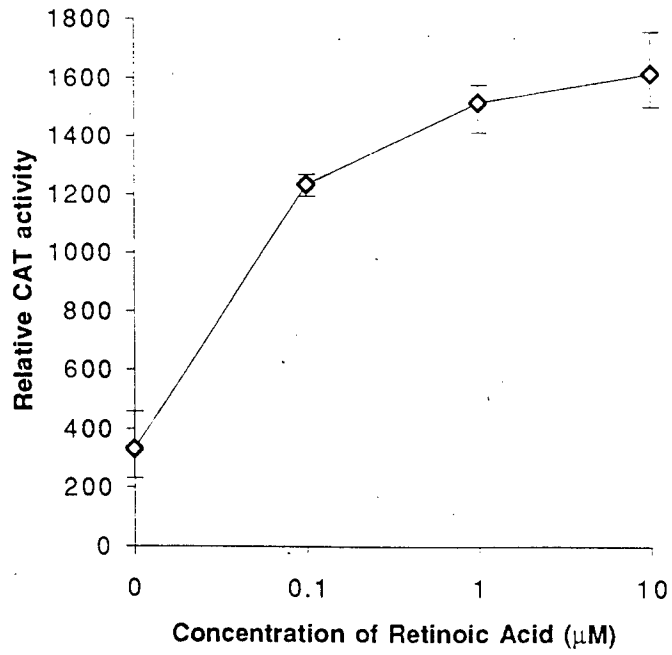


Figure 3

A AG11132

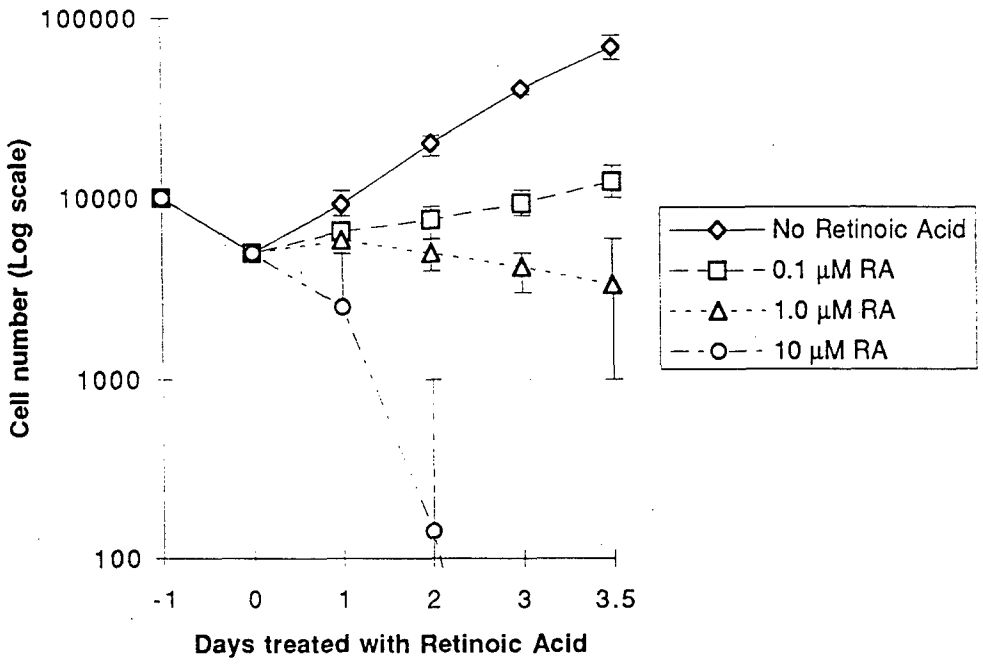


Figure 3

B AG11134

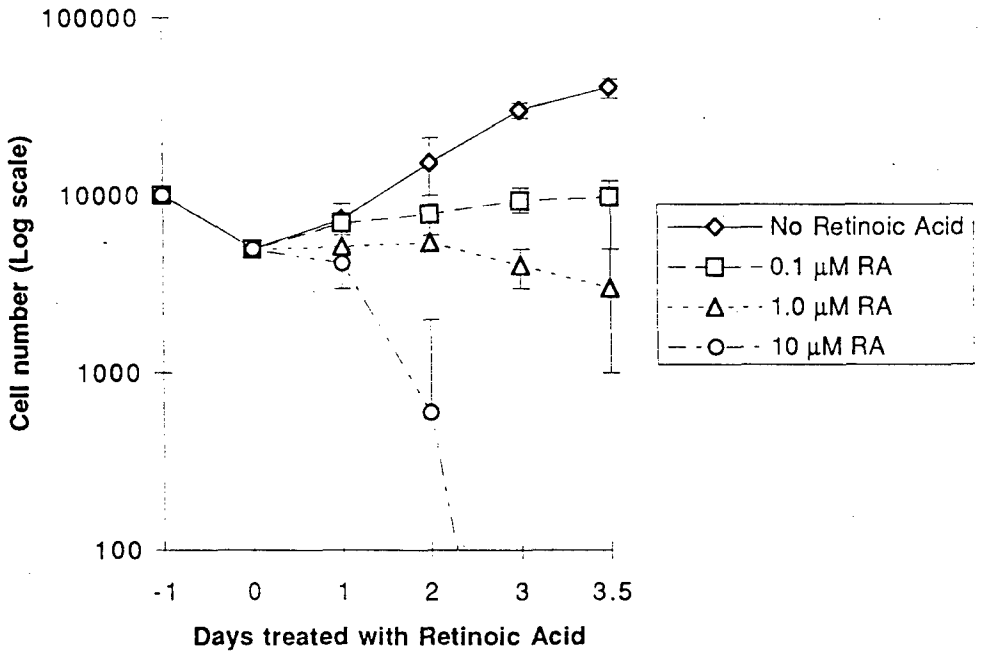


Figure 4

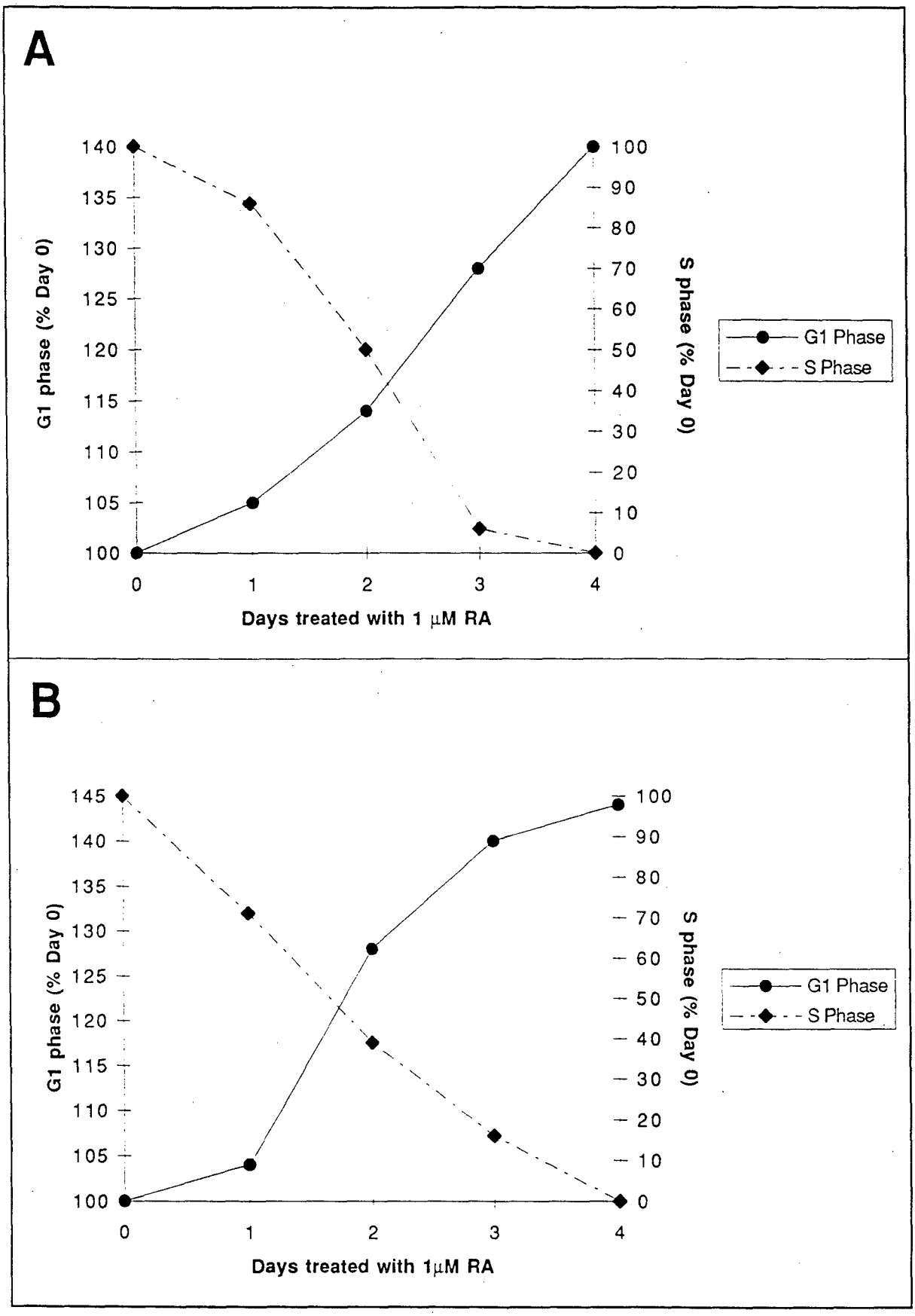

A
140
135
130
125
120
115
110
105
100
G1 phase (% Day 0)
100
90
80
70
60
50
40
30
20
10
0
S phase (% Day 0)
0
1
2
3
4
Days treated with 1 μM RA
G1 Phase
S Phase
B
145
140
135
130
125
120
115
110
105
100
G1 phase (% Day 0)
100
90
80
70
60
50
40
30
20
10
0
S phase (% Day 0)
0
1
2
3
4
Days treated with 1μM RA
G1 Phase
S Phase

Figure 5

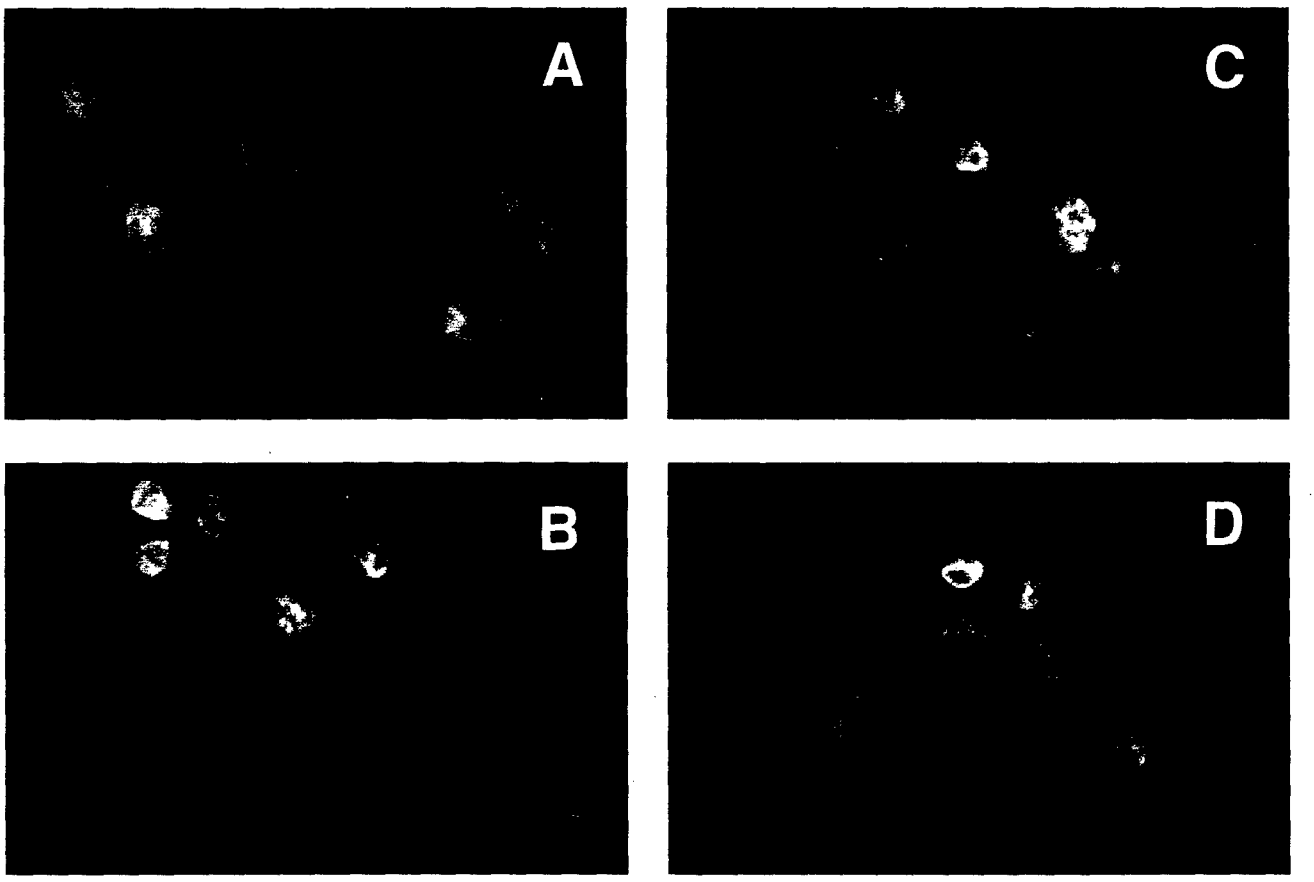


Figure 6

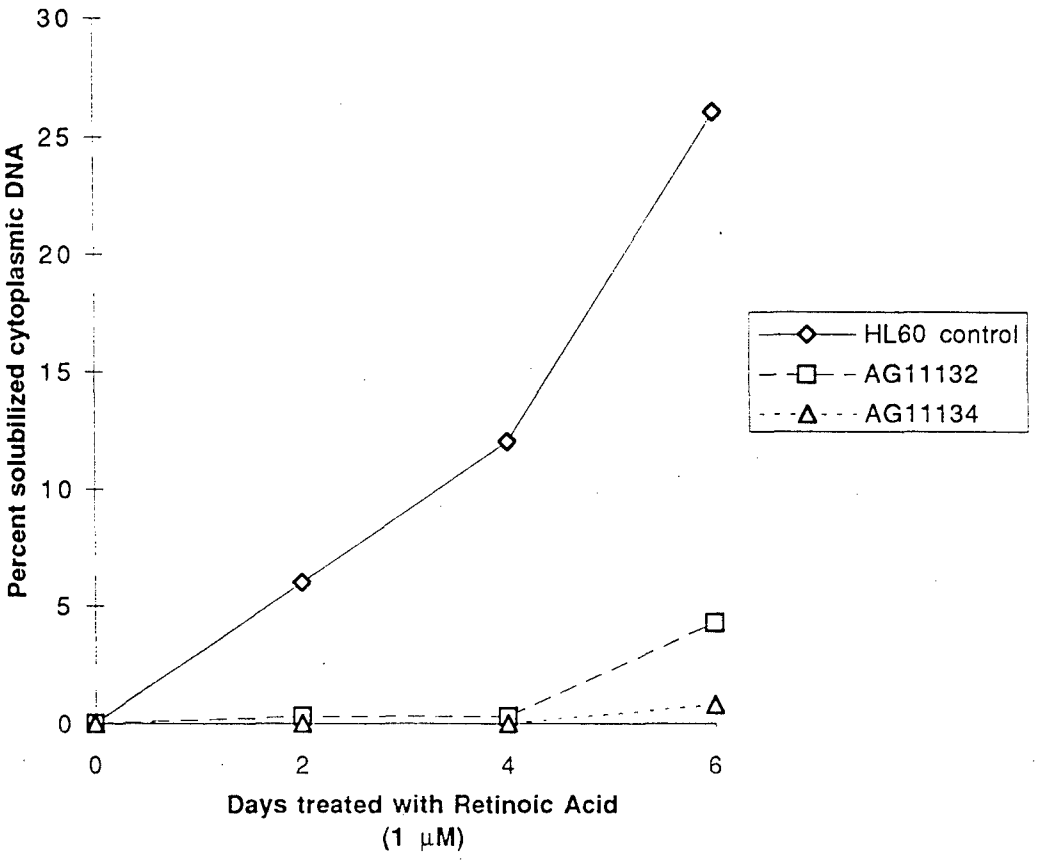

30
25
20
15
10
5
0
Percent solubilized cytoplasmic DNA
0
2
4
6
Days treated with Retinoic Acid
(1 μM)
HL60 control
AG11132
AG11134

Figure 7

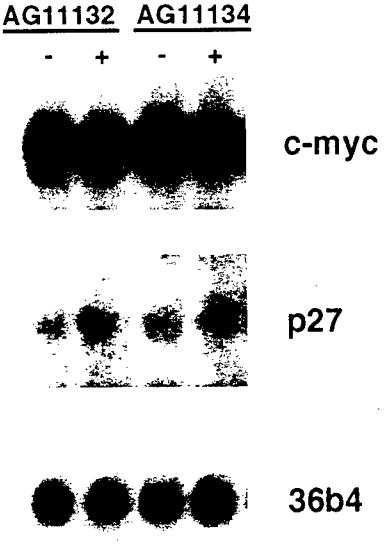


Figure 8

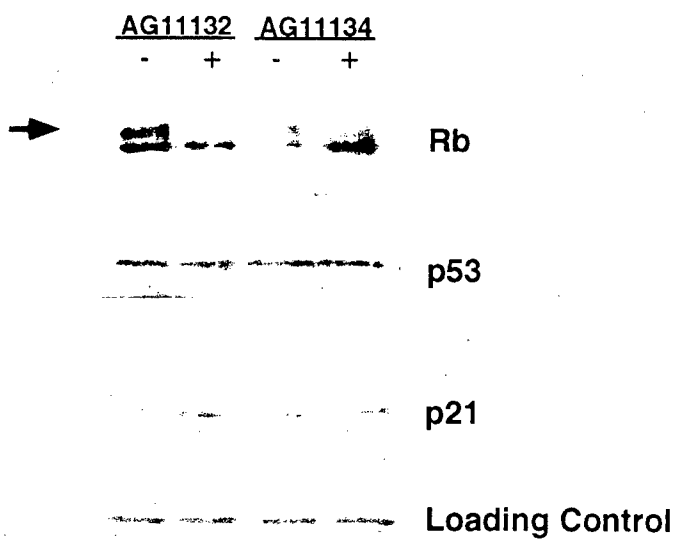


Figure 9

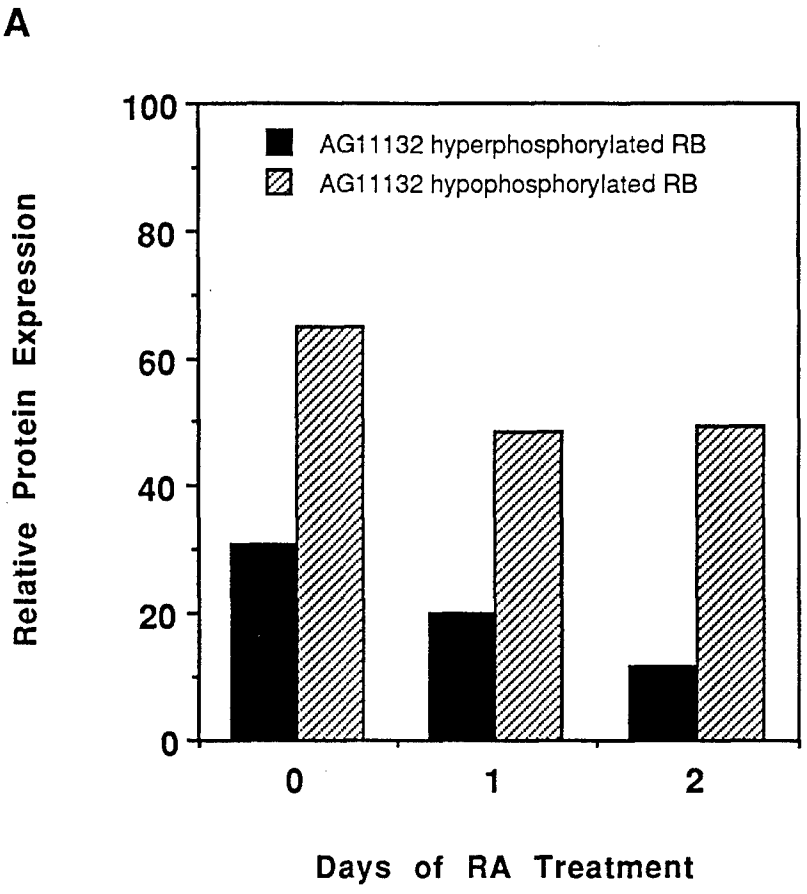
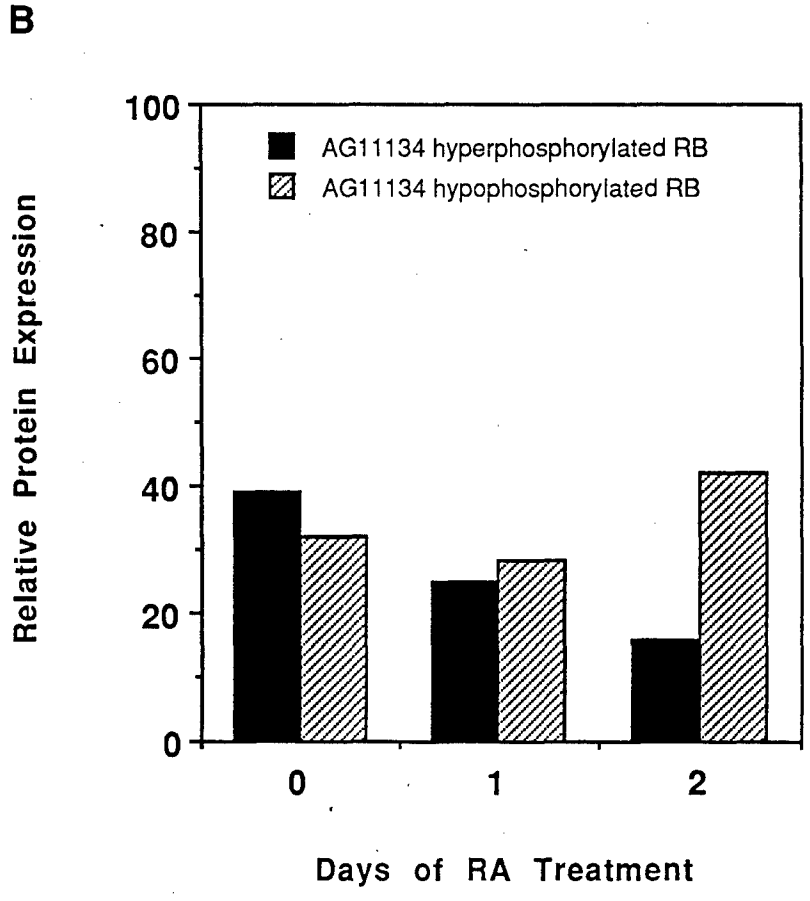


Figure 9



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
DAMD17-94-J-4030	ADB215484
DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864
DAMD17-94-J-4267	ADB216187
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562
DAMD17-94-J-4156	ADB216186
DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management